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APPLICATION NUMBER: ***60/461,016***
FILING DATE: ***April 07, 2003***
RELATED PCT APPLICATION NUMBER: *PCT/US04/10686*

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04/07/03 11055 U.S. PTO

04-08-03

Express Mail Mailing Label No. EV192307027US

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

Attorney Docket No.	RBN-003PR
First Named Inventor	Cheatham

04/07/03 U.S. PTO 60/461016

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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TITLE OF THE INVENTION (280 characters max)

METHODS FOR IDENTIFYING GENES INVOLVED IN GLUCOSE METABOLISM

ENCLOSED APPLICATION PARTS *(check all that apply)*

☒	Specification (*Number of Pages* 64)	☐	CD(s) (Number)
☒	Drawing(s) (*Number of Sheets* 81)	☒	Application Data Sheet (*Number of Sheets* 2)
☒	Claims (*Number of Sheets* 6)	☒	Abstract (*Number of Sheets* 1)
☒	Return Receipt Postcard	☐	Other *(specify)*:

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

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☒ A check in the amount of $80.00.
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FILING FEE AMOUNT $ 80.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☐ No.
☒ Yes, the name of the U.S. Government agency and the Government contract number are: National Institutes of Health: Grant Number R01 CA79907

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2605847

Methods for Identifying Genes Involved in Glucose Metabolism

GOVERNMENT SUPPORT

This invention was made with government support under grant number R01 CA79907 from the National Institutes of Health. The United States Government has certain rights in this invention.

FIELD OF THE INVENTION

The invention provides methods and compositions for identifying and characterizing functionally related gene products associated with glucose metabolism, including pancreatic beta cell and adipocyte function. The invention also provides methods and compositions for identifying and characterizing therapeutic targets and therapeutics for treating diseases associated with altered glucose metabolism and associated cellular dysfunction.

BACKGROUND OF THE INVENTION

Glucose homeostasis is regulated by the coordinated expression of a number of proteins that participate in insulin production and secretion. The pancreatic beta cells of the pancreas sense increased plasma glucose concentrations in the blood and activate a cascade of intracellular reactions leading to the controlled release of insulin from storage granules. Insulin, in turn, controls plasma glucose levels by stimulating glucose-uptake into insulin-sensitive tissues (e.g., skeletal muscle and adipose) and inhibiting hepatic glucose production. Diabetes is a disease characterized by an impairment of insulin control. Type 1 diabetes results from an inability of the pancreatic beta cell to produce insulin, forcing patients to take daily insulin injections to control their blood glucose. Type 2 diabetes is a metabolic disorder in which a patient becomes resistant to insulin's actions, leading to hyperglycemia and hyperinsulinemia. In many cases, Type 2 diabetes is associated with obesity and a sedentary lifestyle. Efforts have been made to

establish pancreatic beta cell lines from adult and embryonic stem cells and to engineer pancreatic beta cell-like cell lines in order to study the metabolic pathways that are activated during development, growth, and maintenance of pancreatic beta cells.

Although some of the cellular pathways involved in glucose metabolism are understood, a number of regulatory aspects of those pathways have not been fully characterized. The identification of mRNAs that are co-regulated with insulin gene expression would provide information about the regulation of genes involved in controlling insulin production and secretion by beta cells of the pancreas. Identification of co-expressed mRNAs would also help identify previously unknown components of the insulin signaling and metabolic pathways in adipocytes and better define those pathways, as well as to provide therapeutic targets for diabetes, obesity, and other diseases characterized by altered glucose metabolism.

SUMMARY OF THE INVENTION

Methods and compositions of the invention are used to isolate proteins and nucleic acids that participate in functionally-related pathways relating to glucose metabolism. In particular, methods and compositions of the invention are used to isolate RNA binding proteins and subsets of mRNAs that participate in insulin production and secretion. The invention is based on the elucidation of the coordinated regulation of multiple genes at the post-transcriptional level by RNA binding proteins. The isolation of a pool of mRNAs associated with an RNA binding protein results in the identification of a functional cluster of mRNAs and related genes, the expression of which is coordinately regulated by the cell. The RNAs bound to a particular RNA binding protein may also possess common primary or secondary structures that mediate binding to the RNA binding protein. RNA binding proteins and mRNAs identified by methods of the invention are useful to elucidate glucose regulatory pathways. In addition, RNA binding proteins are useful to test agents (e.g., therapeutics) for their ability to modulate those pathways and to regulate other genes in that participate in those pathways.

The invention provides insight into pancreatic beta cell processes by identifying mRNAs and proteins that are physically associated with certain RNA binding proteins or mRNP

complexes associated with a phenotype of the cell, e.g., glucose metabolism. For example, the invention provides a ribonomic profile, and methods for identifying and characterizing a ribonomic profile, of the mRNAs, RNA binding proteins and mRNP complex-associated proteins, all of which are associated with a particular mRNP complex.. For example, co-regulated genes participating in glucose metabolism pathway are determined by identifying mRNAs associated with a particular mRNP complex known to be a participant in the pathway. According to the invention, mRNAs or proteins are classified into biologically relevant subsets on the basis of structural and/or functional relationships (*e.g.*, that participate in the same insulin production or secretion pathway, or that facilitate gene expression during growth and development in normal and diseased pancreatic beta cells). In contrast to the static genomics and proteomics approaches to gene characterization and drug discovery, this "ribonomics" approach provides a dynamic snapshot of the flow of genetic information at a given time in the life of a cell or tissue, for example, involved in glucose metabolism, in a normal or diseased state or in response to an environmental influence such as glucose or a drug.

In one aspect, the invention provides methods for identifying comparing differential expression of an RNA binding protein and/or an mRNP complex-associated protein in samples obtained from diseased and healthy individuals. According to the invention, therapeutics are identified based upon targets identified.

In an embodiment, the RNA binding protein is a polypyrimidine tract binding (PTB) protein or another RNA binding protein that is associated with glucose metabolism. Methods of the invention may further comprise the step of identifying as a therapeutic target an mRNA encoding the RNA binding protein and/or an mRNP complex-associated protein, a gene encoding the RNA binding protein and/or an mRNP complex-associated protein, an mRNP complex comprising the RNA binding protein and/or an mRNP complex-associated protein, an mRNA associated with the mRNP complex, and a gene encoding the mRNA associated with the mRNP complex. In an embodiment, gene expression from a differentiated cell, such as an adipocyte or pancreatic beta cell, is compared to that of an undifferentiated cell, such as a preadipocyte. In another embodiment, the cell sample is treated with an agent, such as insulin, a beta-adrenergic agonist, insulin-like growth factor-1 (IGF-1), glucagon-like peptide-1 (GLP-1),

fatty acid, thiazolidinediones and/or glucose and gene expression compared to an untreated cell to identify differential gene expression, which is indicative of a therapeutic target.

In another aspect, the invention provides methods for identifying a gene or gene product involved in a glucose metabolism pathway in a cell by identifying at least one RNA binding protein or mRNP complex-associated protein that is differentially expressed in a normal versus diseased cell (e.g., a cell exhibiting an aberrant phenotype relating to glucose metabolism), isolating an mRNP complex comprising the RNA binding protein or mRNP complex-associated protein and at least one mRNA, and identifying a component of the complex as being involved in a biochemical pathway. In an embodiment, the RNA binding protein is a polypyrimidine tract binding (PTB) protein. In an embodiment, a method of the invention further comprises the step of contacting the cell with an agent that interacts with a component of the biochemical pathway. The agent may inhibit or enhance the biochemical pathway. The pathway may be, for example, an insulin production pathway or a lipogenesis pathway. In an embodiment, the cells are preadipocytes, adipocytes or pancreatic beta cells.

In another aspect, the invention provides methods for identifying an agent that interacts with or regulates a component of a biochemical pathway involved in glucose metabolism by contacting a sample with an agent, preparing a ribonomic profile of the agent-treated sample, and comparing the level of expression of the component in the agent-treated sample to the level of expression of the component in a control sample, wherein a difference in the expression of the component is indicative that the agent is capable of interacting with or regulating the component. The ribonomic profile comprises expression of at least one component of the biochemical pathway associated with at least one mRNP complex. The component may be an RNA binding protein, an mRNA, and/or an mRNP complex-associated protein.

In another aspect, the invention provides methods for assessing the efficacy of an agent as a therapeutic in treating an individual having a disease associated with altered glucose metabolism. Methods comprise the steps of contacting a sample from an individual having a disease with an agent, preparing a ribonomic profile of the agent-treated sample and comparing the level of expression an RNA binding protein or mRNP complex-associated protein in the

agent-treated sample to the level of expression of the RNA binding protein or mRNP complex-associated protein in a control sample, wherein a difference in expression is indicative that the agent is a candidate therapeutic capable of treating the disease.

In another aspect, the invention provides methods for identifying a protein that regulates insulin production by measuring the expression of an RNA binding protein and/or an mRNP complex-associated protein in a pancreatic beta cell sample, treating the pancreatic beta cell sample with glucose, glucagon-like peptide (GLP-1), fatty acid or a thiazolidinedione, and measuring expression of the levels of RNA binding protein and/or an mRNP complex-associated protein after treatment. The difference in the expression of the RNA binding protein and/or an mRNP complex-associated protein after treatment compared to expression before treatment is indicative that the RNA binding protein and/or an mRNP complex-associated protein regulates insulin production.

In another aspect, the invention provides methods of identifying gene products co-regulated with preproinsulin mRNA by isolating an RNA binding protein or mRNP complex-associated protein and identifying a component of the mRNP complex (e.g., mRNA, RNA binding protein, and/or mRNP complex-associated protein).

In another aspect, the invention provides methods for identifying a protein that regulates insulin production. The method comprises the steps of contacting a pancreatic beta cell sample with at least one probe that is capable of binding to at least one protein, wherein the protein is capable of binding to the 3' UTR or 5' UTR of a preproinsulin mRNA, separating the probe from the protein, and identifying the protein. In an embodiment, the probe comprises the sequence 5'-gaauaaaaccuuugaaagagcacuac-3' or 5'-cccaccacuacccuguccaccccucugcaaug-3'.

In another aspect, invention provides isolated compositions of matter, such as mRNP complexes, identified by the above methods. The composition of matter may be an mRNP complex associated with diabetes, wherein the mRNP complex comprises a polypyrimidine tract binding (PTB) protein and at least one mRNA associated with the polypyrimidine tract binding protein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A is a schematic outlining a strategy for the isolation of an RNA binding protein, RBP1, using a RiboTrap™ assay, followed by the isolation and identification of mRNAs associated with PTB, using the RAS™ assay.

Figure 1B is a schematic outline a strategy for the isolation of a 5' untranslated region-binding RNA binding protein and a 3' untranslated region-binding RNA binding protein using a RiboTrap™ assay, followed by the isolation and identification of mRNAs associated with PTB, using the RAS™ assay.

Figure 2 is a schematic for the stimulus-coupling mechanism of insulin secretion in pancreatic beta cells.

Figure 3 is a schematic overview of the data flow for analyzing and interpreting microarray results from comparative RNA binding protein expression and/or mRNP complexes.

Figure 4 is a schematic overview of the RiboTrap™ assay and the Ribonomic Analysis System (RAS™) assay.

Figure 5 is a schematic overview of the target discovery process using RNA binding proteins and mRNP complexes.

Figure 6 is a schematic overview of a RiboTrap™ assay. An mRNA encoding an RNA binding protein (RBP1 or RBP2) of interest tagged with a ligand such as MSII coat protein (CP) binding site (RNA stem loops) is introduced into a cell by transfection and expressed. The tag allows for recovery of the mRNA with its attached RNA binding protein. A binding partner for the ligand such as CP antibody is used to immunoprecipitate the tagged mRNA and its associated RNA binding proteins.

Figure 7 is a Western blot illustrating the *in vitro* verification that PTB specifically binds the 3'UTR and not the 5'UTR of the insulin mRNA from INS-1 cell lysates. Lanes 1 and 2: no oligo; Lanes 3 and 4: control oligo; lanes 5 and 6: 5' UTR oligo; Lanes 7 and 8: 3' UTR oligo.

Figure 8 illustrates glucose-regulated RBP binding to preproinsulin mRNA.

Figure 9 is a schematic overview of target discovery of primary adipocytes.

Figure 10 is a graphical representation of expression data generated on a human Ribochip™ microarray comparing RNA binding protein expression of adipocytes and preadipocytes. These graphs are output of GeneSpring comparison of data sets showing relative gene expression levels between two conditions or samples.

Figure 11A is a graphical representation of expression data generated on a human Ribochip™ microarray comparing RNA binding proteins that are down-regulated in human adipocytes compared to preadipocytes.

Figure 11B is a graphical representation of expression data generated on a human Ribochip™ microarray comparing RNA binding proteins that are up-regulated in human adipocytes compared to preadipocytes.

Figure 12 is a list of RNA binding proteins that are increased 2-fold or more in adipocytes compared to preadipocytes from lean individuals.

Figure 13A is a graphical representation of expression data generated on a human Ribochip™ microarray comparing the effects of 100nm insulin or 1μM β3 agonist BRL-37344 on RNA binding protein expression over basal levels in human adipocytes from lean individuals.

Figure 13B is a graphical representation of expression data generated on a human Ribochip™ microarray showing the expression of RNA binding proteins that are increased or decreased 1.5-fold or more over basal levels by treatment of adipocytes from lean individuals with 100nm insulin or 1μM β3 agonist BRL-37344.

Figure 14 is a list of RNA binding proteins that are increased 2-fold or more over basal levels after treatment of adipocytes from lean individuals with insulin.

Figure 15 is a list of RNA binding proteins that are increased 2-fold or more in adipocytes compared to preadipocytes from obese individuals.

Figure 16A-16K is a list of 3231 mRNAs generated from RAS™ analysis of the PTB RNA binding protein.

Figure 17 identifies the ion channel proteins associated with PTB that have not previously been associated with the glucose metabolic pathway.

Detailed Description

The invention provides methods for mining and characterizing the cellular ribonome in cells that participate in glucose metabolism. Specifically, the invention allows elucidation of cellular gene regulation by identifying and measuring mRNAs and proteins that are functionally co-associated with mRNP complexes that participate in glucose metabolism.

Methods of the invention compose identifying components of mRNP complexes. The identified components are useful for diagnosing, monitoring, and assessing the metabolic or disease state of a cell. Components of mRNP complexes can also be used to identify potential therapeutic targets as well as for assessing the efficacy or toxicity of potential therapeutics. Moreover, the invention provides methods for identifying and characterizing structurally and/or functionally related gene products, and to elucidate features of biological pathways.

Generally, an mRNP complex comprises various components that may include, but are not limited to, at least one RNA binding protein, at least one associated or bound mRNA, and at least one associated or bound protein (*i.e.*, an mRNP complex-associated protein). An mRNP complex may also comprise other associated or bound molecules (*e.g.*, carbohydrates, lipids, vitamins, etc.). A component associates with an mRNP complex if it binds or otherwise attaches to the mRNP complex with a Kd of about 10^{-6} to about 10^{-9}. In a preferred embodiment, the component associates with the complex with a Kd of about 10^{-7} to about 10^{-9}. In a more preferred embodiment, the component associates with the complex with a Kd of about 10^{-8} to about 10^{-9}.

By isolating an mRNP complex in a cell, and, preferably, identifying the components of the mRNP complex and the gene precursors and gene products of those components, a ribonomic gene expression profile is generated. By identifying the mRNA components of a cellular ribonome, the cellular transcriptome is broken down into a series of subprofiles that together are used to define the gene expression state of a cell or tissue. The associated or bound mRNAs are categorized into distinct subsets based on their association with a particular RNA binding protein, mRNP complex-associated protein, or common structural feature. Ribonomic profiles differ from cell sample to cell sample, depending on a variety of factors including, but not limited to, the differentiation status of the cell, the species or tissue type of the cell, the developmental stage of the cell, the pathogenicity of the cell (*e.g.*, if the cell is infected, is expressing a deleterious gene, is lacking a particular gene, is not expressing a particular gene, or is overexpressing a particular gene), the specific ligands used to isolate the mRNP complexes, the various conditions affecting the cell (*e.g.*, environmental, apoptotic or stress states, and disease or other disorder), as well as other factors known to practitioners in the art.

Identification and Isolation of mRNP Complexes

Messenger RNP complexes associated with glucose metabolism are identified by generating an expression pattern of RNA binding proteins in stimulus-challenged cells. The expression profiles enable the identification of selectively-expressed and/or selectively-regulated RNA binding proteins for further analysis. For glucose-regulated processes, mRNA samples from islets, MIN-6, and INS-1 cells are subjected to different concentrations of glucose for varying periods of time. Glucose alters the expression of RNA binding proteins involved in glucose-regulated functions in the pancreatic beta cell, so that tissue-specific\enriched expression of RNA binding proteins can be detected. Analysis of non-beta cell mRNA samples (e.g., NIH3T3) provide a baseline comparison.

A microarray-based assay utilizing a custom-designed oligo-microarray (RiboChip™ MWG Biotech (High Point, NC) containing 1400 features representing genes, the protein products of which are reported to have RNA binding properties or RNA binding motifs. Also included on the array are control features (total of 17) that provide information on

specificity, labeling and hybridization efficiency, sensitivity and normalization between experiments. The mRNA samples to be analyzed are prepared from whole cells, amplified if necessary, and processed for microarray hybridization. Briefly, total mRNA, or if necessary amplified mRNA, from the cell samples is labeled using amino allyl coupling chemistries (CyScribe Post Labeling Kit, Amersham). Amino allyl labeling reduces the fluorescent labeling bias observed with direct incorporation of cy5 and cy3-labeled nucleotides. After generation of the single stranded amino allyl product, the probe is fluorescently labeled with either cy3 or cy5. If multiple probes are to be used for a single array, they are dried down and are resuspended in nuclease free water containing control probes (e.g., Cot-1 and Arabidopsis DNAs) and hybridization buffer. After denaturing, probes are then incubated with a mouse RiboChip™ microarray. Hybridization is carried out routinely at 65°C for 18 hours. The arrays are sequentially washed with 0.5x SSC/0.1% SDS and then 0.6x SSC. Data are gathered from the array using a GenePix 4000B scanner and associated software. The data are then processed further for clustering\profiling and graphical representation using GeneSpring. In addition to providing descriptive measures of data quality, statistical techniques are used to group or cluster genes based on similarities and differences in their expression profiles. A flowchart summarizing the data analysis is provided in Figure 3. These methods enable genes with unique or "interesting" expression profiles to be quickly identified from among the thousand genes profiled in a single experiment. In one such application of clustering, the expression profiles for 24 genes encoding RNA binding proteins from 33 normal human tissues were clustered (Pearson) and represented as a dendogram using GeneSpring (Silicon Genetics). Each column represents a distinct RNA binding protein gene and each row represents a distinct tissue sample. The data was normalized to the median expression value. Yellow indicates expression within 10-fold of the median value; blue indicates expression more than 10-fold below the median value; and red indicates expression more than 10-fold above the median value. This representation enabled groups of, and distinctions between, expression profiles to be quickly identified.

RiboTrap™

RiboTrap™ is an alternative, more directed, means for identifying RNA binding proteins. RiboTrap™ combines a biochemical and molecular biological approach for isolating an unknown RNA binding protein or set of RNA binding proteins that interact with an mRNA of interest. This involves several different approaches including the use of 1) epitope-tagged binding elements as affinity reagents for *in vitro* isolation of RNA binding proteins and 2) expression of an epitope-tagged mRNA in cell culture models. Approaches for isolating the glucose-responsive RNA binding protein (s) in pancreatic beta cells and RNA binding proteins interacting with Nkx6.1 mRNA using current technologies are described in more detail below. RiboTrap™ is useful when it is necessary to first identify an RNA binding protein on a specific mRNA. RiboTrap™ methods are described in more detail in Example 2.

An mRNP complex is isolated from a natural biological sample such as an islet, a pancreatic beta cell, an adipocyte, or a preadipocyte, or a population of cells. The population of cells may contain a single cell type. Alternatively, the population of cells may contain a mixture of different cell types from either primary or secondary cultures or from a complex tissue such as an islet or tumor.

Freshly prepared islets are the most physiologically relevant *in vitro* model system for examining glucose-responsiveness and endocrine pancreas functions. Preparation of islets and islet mRNA samples are prepared as described in Example 2. To identify RNA binding proteins that undergo changes in expression, mouse islets are incubated under conditions of low (2.8 mM) or high (16.6 mM) glucose for various periods of time (up to 24 hours). Total mRNA is prepared using Trizol according to standard methods. In some cases where samples are limiting, it may be necessary to amplify the mRNA. This is routinely accomplished using the RiboAmp™ kit (Arcturus).

Islets are composed of four unique cell-types α, β, γ, and δ. All RNA binding proteins expressed in islets can be profiled or RNA binding proteins from pancreatic beta cell line and an alpha cell line can be prepared. The use of cell lines is also advantageous for easier manipulation of conditions as well as providing a culture system for future validation of

targets. The mouse-derived pancreatic beta cell line, MIN-6, may be used. Compared to available pancreatic beta cell lines, MIN-6 cells respond well to glucose and have been used for numerous studies for glucose-stimulated insulin secretion and proinsulin biosynthesis. As with the islets, the MIN-6 cells are exposed to low and high glucose concentrations for various periods of time and mRNA prepared and processed for microarray analysis on the mouse RiboChip™.

The alpha cell line, α-TC1.6, may be used for comparison to islets and MIN-6 cells. These cells express mRNA for Nkx6.1, but do not express Nkx6.1 protein. In contrast, pancreatic beta cells express both mRNA and protein for Nkx6.1. Current evidence supports a role of RNA binding proteins in this restrictive expression during islet development.

In one embodiment, the mRNP complex is isolated from a cell sample in which the expression of a component of an mRNP complex has been altered, *e.g.*, induced, inhibited, or over-expressed. In another embodiment, a particular mRNP complex or component or precursor for one or more components of the mRNP complex has been introduced into the sample or has been genetically altered. Introduction of the one or more mRNP complex components may occur by infection, transformation, or other similar methods known in the art. In one embodiment, an expression vector expressing one or more components of an mRNP complex is transfected into the cell. Suitable vectors include, but are not limited to, recombinant vectors such as plasmid vectors or viral vectors. The component is preferably operatively linked to appropriate promoter and/or enhancer sequences for expression in the cell. In an embodiment of the invention, a specific cell type is engineered to contain a cell type-specific or inducible gene promoter that drives expression of an RNA binding protein. A ligand, such as an antibody that is specific for this RNA binding protein, may immunoprecipitate the RNA binding protein, with its attached or associated mRNAs, from a tissue extract containing the cell type of interest. The RNAs are then identified to form the expression profile of that cell type or isolated for further research, as described herein.

Alternatively, the cell sample may contain a knock out cell line or knock out organism that either does not express a component of the mRNP complex or expresses decreased levels of

the component. Preferably, the knock out cell line or knock out organism does not express a particular RNA binding protein, an mRNA associated with the mRNA complex or RNA binding protein, or an mRNP complex-associated protein.

In a preferred embodiment, the nucleic acid encoding the mRNP complex component is tagged (*e.g.*, a tagged RNA binding protein) in order to facilitate the separation, observation and/or detection of the components. Accessible epitopes may be used or, where the epitopes on the components are inaccessible or obscured, epitope tags on ectopically expressed recombinant proteins may be used. Suitable tags include, but are not limited to, biotin, the MS2 protein binding site sequence, the U1snRNA 70k binding site sequence, the U1snRNA A binding site sequence, the g10 binding site sequence (Novagen, Inc., Madison, WI), and FLAG-TAG® (Sigma Chemical, St. Louis, MO). For example, a transformed cell containing a transfected vector directing the expression of a tagged RNA binding protein can be mixed with other cell types or can be implanted in an animal or human subject. In an embodiment, a ligand, such as an antibody or antibody fragment, that is specific for the tag is used to immunoprecipitate the tagged RNA binding protein with its associated mRNAs from a tissue extract containing the transformed cell. The mRNP complexes and associated RNAs can then be identified either to form an expression profile for that cell type for further analysis.

The expression of one or more mRNP complex components may be altered by contacting or treating the cell sample with a known or test compound. The compound may be, but is not limited to, a protein, a nucleic acid, a peptide, an antibody, an antibody fragment, a small molecule, or an enzyme. Where the compound is a nucleic acid, the nucleic acid may be an antisense nucleic acid, a ribozyme, an RNAi, an aptamer, a decoy nucleic acid, or a competitor nucleic acid. In one embodiment, the compound may alter the expression of an mRNP complex component through competitive binding. A compound may inhibit binding between an RNA binding protein and an mRNA, between an RNA binding protein and an mRNP complex-associated protein, or between an mRNA and an mRNP complex-associated protein, for example. In another embodiment, the cell sample is infected with a pathogen, such as a virus, bacteria, prion, fungus, parasite, or yeast, to alter expression of one or more mRNA complexes.

While any method for the isolation of an mRNP complex may be used in the present invention, the methods disclosed in co-pending U.S. Application Nos. 09/750,401; 10/238,306; and 10/309,788 are preferred, the entire disclosures of which are hereby incorporated by reference. The *in vivo* methods for isolating an mRNP complex involve contacting a biological sample that includes at least one mRNP complex with a ligand that specifically binds a component of the mRNP complex. For example, the ligand may be an antibody, a nucleic acid (*e.g.*, an antisense, aptamer, or RNAi molecule), or any other compound or molecule that specifically binds the component of the complex. In certain embodiments, the ligand is obtained by using the serum of a subject that has a disorder known to be associated with the production of mRNP complex-specific antibodies or proteins. Examples of these disorders include autoimmune disorders and a number of cancers. In certain embodiments, the ligand is tagged with another compound or molecule in order to facilitate the separation, observation or detection of the ligand. In one embodiment of the invention, the ligand is epitope tagged, as described in the art.

In an embodiment, the mRNP complex is separated by binding the ligand (now bound to the mRNP complex) to a binding molecule that specifically binds the ligand. The binding molecule may bind the ligand directly (*e.g.*, a binding partner specific for the ligand), or may bind the ligand indirectly (*e.g.*, a binding partner specific for a tag on the ligand). Suitable binding molecules include, but are not limited to, protein A, protein G, and streptavidin. Binding molecules may also be obtained by using the serum of a subject suffering from a disorder such as an autoimmune disorder or cancer. In an embodiment, the ligand is an antibody that binds a component of the mRNP complex via its Fab region and a binding molecule binds the Fc region of the antibody.

In an embodiment, the binding molecule is attached to a support (*e.g.*, a solid support such as a bead, well, pin, plate, or column). Accordingly, the mRNP complex is attached to the support via the ligand and binding molecule. The mRNP complex may then be collected by removing it from the support (*e.g.*, by washing or eluting it from the support using suitable solvents and conditions that are known to a skilled artisan).

In certain embodiments of the invention, the mRNP complex is stabilized by cross-linking prior to binding the ligand thereto. Generally, cross-linking involves covalent binding (*e.g.*, covalently binding the components of the mRNP complex together). Cross-linking may be carried out by physical means (*e.g.*, by heat or ultraviolet radiation), or chemical means (*e.g.*, by contacting the complex with formaldehyde, paraformaldehyde, or other known cross-linking agents), methods of which are known to those skilled in the art. In other embodiments, the ligand is cross-linked to the mRNP complex after binding to the mRNP complex. In additional embodiments, the binding molecule is cross-linked to the ligand after binding to the ligand. In yet another embodiment, the binding molecule is cross-linked to the support.

The methods of the invention allow for the isolation and characterization of a plurality of mRNP complexes simultaneously (*e.g.*, "*en masse*"). For example, a biological sample is contacted with a plurality of ligands each specific for different mRNP complexes. A plurality of mRNP complexes from the sample bind the appropriate specific ligands. The plurality of mRNP complexes are then separated using appropriate binding molecules, thereby isolating the plurality of mRNP complexes. The mRNP complexes and the mRNAs contained within the mRNP complexes are then characterized and/or identified by methods described herein and known in the art. Alternatively, the methods of the invention are carried out on a sample numerous times and the mRNP complexes are characterized and identified in a sequential fashion, with each iteration utilizing a different ligand.

Amplification of the mRNA isolated according to the methods of the invention and/or the cDNA obtained from the mRNA is not necessary or required by the present invention. However, the skilled artisan may choose to amplify the nucleic acid that is identified according to any of the numerous nucleic acid amplification methods that are well-known in the art (*e.g.*, polymerase chain reaction (PCR), reverse transcriptase polymerase chain reaction (RT-PCR), quantitative polymerase chain reaction (QT-PCR), or strand displacement analysis (SDA)).

Analysis of Isolated mRNP Complexes - RAS™ Analysis

The present invention provides methods for assessing the metabolic or gene expression state of a cell such as a pancreatic beta cell. Following isolation of at least one mRNP complex, the level of expression of at least one mRNA associated with the mRNP complex and/ or at least one mRNP complex-associated protein is determined. In an embodiment, the level of expression of the mRNA(s) or the mRNP complex-associated protein(s) on a particular mRNP complex provides a subprofile that is indicative of, for example, the gene expression of a subset of functionally related gene products relating to pancreatic beta cell function or glucose metabolism. In an embodiment, a subset of mRNAs associated with a particular mRNP complex identifies a ribonomic subprofile that is characteristic of a functional RNA network or biological pathway relating to glucose metabolism. The collection of mRNA subsets for any particular cell or tissue sample constitutes a gene expression profile, and, more specifically, a ribonomic gene expression profile, for that cell or tissue. It will be appreciated that ribonomic profiles may differ from cell to cell as described previously. Thus, the ribonomic profile of a cell can be used as an identifier for the cell and can be compared with profiles or subprofiles of other cells.

Approaches for Isolation of Functional Clusters of mRNAs

The preferable technique for isolating functional clusters associated with glucose stimulated insulin secretion and β-cell homeostasis is *in vivo* RAS™, whereby affinity reagents specific to the RNA binding protein of interest are used to isolate and purify the endogenous RNA binding protein complexed with its unique repertoire of mRNAs (a "functional cluster"). If this technique for isolating endogenous RNA:protein complexes is not feasible, for example due to ineffective affinity reagents for immunoprecipitation of the in tact endogenous complex, other complementary approaches to determine the mRNA composition for an RNA binding protein can be used, including: *in vivo* RAS™ with tagged RNA binding proteins and *in vitro* RAS™

In vitro RAS™

In short, RNA binding proteins were cloned by PCR and the sequence verified and expressed in *E. coli* as GST fusion proteins. Following purification, the GST-RNA binding protein was attached to glutathione Sepharose beads and exposed to mRNA preparations to

assess their abilities to selectively retain discreet mRNA pools. In general, two types of mRNA preparations were used, cleared cytoplasmic lysates and purified cytoplasmic RNA. Purified cytoplasmic RNA were used to directly identify mRNAs that encode *cis* binding elements for the RNA binding protein. Using αCP1 that binds to and stabilizes the α–globin mRNA, it was recently shown that the use of cellular lysates containing both RNA and protein improves the specificity of the RNA binding protein:RNA interaction likely due to the presence of auxillary factors that modulate binding. Cytoplasmic lysates therefore offer the best source to identify mRNAs being regulated by RNA binding protein (e.g., PTB) in INS-1 cells. mRNA retained by the individual GST- RNA binding proteins was profiled by combined microarray and QPCR analyses. For additional glucose-regulated, tissue specific RNA binding proteins, comparisons are made between mRNA pools retained using purified (naked) RNA and those selected from cytoplasmic lysates. UTR sequences are aligned to search for obvious consensus elements in the retained mRNA pools, and a small number (e.g., 5-10 UTRs) are initially evaluated to confirm direct binding by biotinylated oligonucleotide-affinity chromatography (as described for RiboTrap™). Over 50 RNA binding proteins have been identified and *in vitro* RAS™ has been performed on three of these: PTB, ACO-1 and HNRNP-K. Each of these RNA binding proteins binds mRNA from both purified RNA and lysate preparations. The bound mRNA species for each RNA binding protein is determined by array analysis and QPCR.

Affinity-tagging of RBPs *in vivo*.

The multicomponent nature of RNPs can interfere with efficient immunoprecipitation due to seclusion of reactive polypeptide epitopes. As a consequence, finding appropriate affinity reagents and optimal conditions for immunoprecipitation of endogenous complexes can be challenging. In the absence of appropriate affinity reagents or when endogenous complexes cannot be isolated, mRNAs associated with individual RNA binding proteins in a cell are identified by using RNA binding proteins tagged with one of several generic epitopes such as Flag, AU1, or T7. The binding epitopes are expressed on the N- or C-terminus of PTB and other RNA binding proteins and introduced into an appropriate cell line for expression. Pooled cell lines are generated by selection (e.g., zeocin) and screened for stable

expression of the tagged RNA binding proteins. Commercially available antibodies (e.g., α-T7) are used to immunoprecipitate RNPs from INS-1 cells following mock or glucose treatment. As a positive control, tagged PABP1 (poly A binding protein), which is known to bind virtually all polyadenylated mRNAs, is constructed and selected into INS-1 cells for parallel immunoprecipitation of RNPs. Messenger RNA pools isolated following low and high glucose treatment of the individual INS-1 cell lines (pooled lines) are evaluated by microarray analysis and selective QPCR confirmation. he use of a tagged-PTB isdvantageous in that the functional cluster associated of the tagged-PTB can be directly compared with that isolated using the commercially available monoclonal antibody to PTB (Zymed). This allow for validation of the endogenous PTB cluster as well as assessment of the mRNA binding characteristics of tagged-PTB.

Immunoprecipitation of endogenous complexes (RAS™)

By far, the most powerful method available to profile endogenous RNP complexes from cells or tissue utilizes immunoprecipitation of endogenous RNPs from cell lysates and characterization of mRNA content. Polyclonal anti-peptide and\or full-length protein antibodies, monoclonal antibodies, or recombinant antibody libraries may be used. Optionally, more than one peptide antigen may be chosen based on analysis of the protein sequence using software for antigenic determination (Antheprot; uses Parker and Wellington algorithms), followed by a Blast P search in NCBI to ensure that the designed peptide is not significantly homologous to another protein. Peptides are selected from regions thought to lie outside the RNA binding domain to enrich for epitopes that are more likely to be exposed in the RNP complex. 15-25 amino acid peptides are contracted externally for synthesis and conjugation to KLH followed by immunization of rabbits for polyclonal antibody production.

Antibodies to individual RBPs are used to isolate associated mRNAs at any given disease state or under experimental conditions. In contrast to the tagged RBP approach described above, isolation of endogenous RNPs does not require perturbation of the system (e.g., transfection and selection of cell lines expressing tagged RBP) prior to analysis. However, the rate-limiting step in this analysis is acquisition of antibodies specific for individual RNA binding proteins which can immunoprecipitate intact endogenous RNPs. Conditions have been defined using a commercial antibody for PTB that effectively

immunoprecipitates PTB containing RNPs from INS-1 cells. It is clear that with careful attention to antibody selection and optimization of lysis and washing conditions, endogenous RNPs can be routinely isolated from cells and tissues. Once a candidate RNA binding protein has been chosen for RAS™, polyclonal antibodies are made using the recombinant RNA binding proteins prepared as described for *in vitro* RAS™.

Full-length RNA binding protein genes are PCR amplified out of appropriate cDNA libraries and cloned into expression vectors (pGEX or pDEST17 6X-His) for bacterial expression purification and antibody production. Antibodies are affinity-purified, characterized, and optimized for immunoprecipitation of the protein and its associated RNA binding proteins/mRNA complex. The ability of the antibody to precipitate ribonucleic acids in general is determined by a rapid, high-throughput analysis using a 2100 BioAnalyzer (Agilent). Non-immune controls include previously characterized RNA binding protein antibodies run in parallel as negative and positive controls, respectively. Specific antisera that are able to immunoprecipitate the RNA binding protein\mRNA complex are used for further analysis. Nuclei-free cytosolic extracts are prepared essentially as described from cells (or tissue) that have been exposed to various experimental conditions (e.g., low and high glucose). For immunoprecipitation of the RNA binding protein /mRNA complex, specific antibodies are pre-bound to protein A beads, blocked with bovine serum albumin and washed extensively. After a final wash in lysis buffer, cell extracts are added. Incubation times and temperatures are optimized for each anti- RNA binding protein. The complexes are washed extensively under nuclease-free conditions. The antibody-mRNP complex is then disrupted with denaturing buffer RLT containing guanidine thiocyanate, and mRNA purified using Qiagen RNA isolation column chromatography. The purified mRNA is then processed for microarray analysis on contemporary human or rodent microarrays (depending on the cell or tissue source) comprised of features (10,000-30,000 genes) representing up-to-date genomic content (Agilent or MWG). Genes observed at 'detectable' levels that are present in each of the experiments is considered a component in that RBP's complex and its relative fold-enrichment above a total RNA microarray analysis is determined. The presence of the candidate genes and their relative fold-enrichment over total RNA are verified and more accurately quantified by Q-PCR using sequence-specific primers. Routinely, genes enriched

at a LEVEL ≥2-fold above total RNA sample are considered members of that cluster. The above combination of *in vitro* and cell culture based approaches to map mRNA pools accurately defines the mRNA content of selected RNPs. The combined use of these integrated techniques offers an unparalleled algorithm to define the cell's organization of coordinately regulated mRNAs.

Supporting analytic and bioinformatic platform

The mRNA samples to be analyzed are prepared from various cell and tissue-types by RNA extraction with RNeasy™ (Qiagen, Inc.), quantified by absorbance (A_{260}), and stored at -80°C until use. RNA samples are routinely evaluated using an Agilent Bioanalyzer 2100 which provides a rapid and quantitative measure of 28S and 18S ribosomal RNA integrity. RNA is labeled with or without amplification through generation of cDNA by reverse transcription in the presence of amino allyl-dUTP followed by direct coupling to Cy3 or Cy5 fluorescent dyes (CyScribe Post Labeling Kit, Amersham). If RNA quantities are limiting (< 10 μg), nucleic acid amplification is accomplished with reverse transcription, cDNA tailing, and PCR amplification procedures prior to Cy3/Cy5 coupling. If amplification of RNA from an RNA binding protein complex is needed, the total RNA sample is also amplified. Hybridization is carried out routinely at 65 °C for 18 hours. The arrays are sequentially washed with 0.5X SSC/0.1% SDS and then 0.6X SSC. Microarray slides are read by an Axon 4000B scanner, images acquired by GenePix 4.0 (Axon), and features extracted with Imagene V4.2 (Biodiscovery). Data analysis, including normalization (lowess), centralization, and scaling is accomplished using GeneSpring 4.2.1(Silicon Genetics). Differentially expressed genes are identified using a combination of methods for assessing variation in expression level between conditions. These include t-tests and ANOVA analysis with multiple testing corrections, fold difference filtering, and clustering techniques. These methods enable genes with unique or "interesting" expression profiles to be quickly identified from among the thousand genes profiled in a single experiment. Genes within a cluster are chosen based upon relative enrichment of expression compared to total RNA samples. Routinely, genes enriched at a LEVEL ≥2-fold above total RNA sample are considered members of that cluster (e.g., PTB cluster from Rat INS-1 cells contains 3231 genes using 2-fold enrichment over total RNA sample as the statistical cut-off (Figure 16A-16K). Data was

generated using rat INS-1 cells (BetaGene, Inc.) and MWG 10K Rat Pan Arrays (cat# 2250-000000). This analysis revealed a highly enriched (>5-fold) subset of approximately 450 genes. The normalized intensities of many of the 650 genes were altered (>2-fold) in the clusters isolated from cells treated with 15mM glucose whereas the same genes in the total RNA analysis were unchanged. This suggests that glucose could regulate the appearance of many mRNAs into or out of the cluster. In support of the functional cluster hypothesis, numerous predicted genes were highly enriched in the PTB-cluster and the presence of many of these was regulated by glucose. Included in this list are mRNAs for Glut2, glucokinase, phosphofructokinase, Kir6.2 (the ATP-sensitive K+-channel), SUR1 (sulfonylurea receptor 1), L-type Ca2+-channels, acyl-coa carboxylase and preproinsulin. In addition, and importantly, approximately 10% of the 450 genes in the PTB cluster had normalized intensity values at or below detectable levels when analyzed by normal array analysis of total mRNA samples. Thus, the ability to isolate the PTB cluster, purify and identify its associated mRNAs lead to the identification of very low abundant genes that most likely would have been missed or ignored in a normal array analysis. The ability to isolate the PTB cluster, enrich for a unique subset of genes, their regulated appearance in the cluster and identification of very low abundant genes supports the hypothesis regarding the role of rbps in gene/protein expression and their utility to obtain novel target and cellular pathway information. Expression of all candidate mRNAs in an RNP complex chosen for further downstream analysis are verified at the mRNA level by Q-PCR using gene specific primers.

Accordingly, the present invention provides diagnostic methods for assessing the cell types present in a sample or a population of cells such as pancreatic beta cells, adipocytes, or preadipocytes. The method involves isolating at least one mRNP complex and detecting the expression of at least one component of the mRNP complex, wherein the at least one component is specific for a certain cell type, so that the detection of the expression of the component is indicative of the presence of the cell type in the population of cells. The component may be specific for a certain cell type within an entire sample (*e.g.*, tissue or organism) or within the population of cells. The sample or population of cells may be, for example, a tumor, a tissue, a cultured cell, a body fluid, an organ, a cell extract or a cell lysate. The methods of the invention may also be used to determine the cell types present in a population of cells. Alternatively, cell

type, as used herein, may also refer to a class of cells derived from a particular tissue, a particular species, a particular state of differentiation, a particular disease state, or a particular cell cycle.

An isolated mRNP complex can be examined, in part to determine expression of its components, as a whole, or broken into its components. The mRNP complex can be separated from the ligand as a whole, or the mRNA can be separated from the ligand-RNA binding protein complex, followed by separation of the RNA binding protein from the ligand. Alternatively, if the mRNA is bound to the ligand, the RNA binding protein can be separated from the ligand-mRNA complex, and the mRNA then separated from the ligand. Practitioners in the art are aware of standard methods of separating the components, including washing and chemical reactions. After separation, each component of an mRNP complex can be examined and their identity, quantity, or other identifying factors preferably recorded (*e.g.*, in a computer database) for future reference.

cDNAs can be used to identify complementary mRNAs on mRNP complexes partitioned according to methods disclosed herein. cDNA microarray grids can be used to identify mRNA subsets *en masse*. Microarrays are precisely aligned grids in which each target nucleic acid (*e.g.*, cDNA, oligonucleotide, or gene) has a position in a matrix of carefully spotted cDNAs. Each target nucleic acid examined on a microarray has a precise address that can be located, and the binding can be quantitated. Microarrays may be arranged in a commercially available substrate (*e.g.*, paper, nitrocellulose, nylon, any other type of membrane filter, chip, such as a siliconized chip, glass slide, silicone wafer, or any other suitable solid or flexible support). In addition, mRNAs in a sample can be identified based upon the stringency of binding and washing, a process known as "sequencing by hybridization."

Alternative approaches for identifying, sequencing and/or otherwise characterizing the mRNAs in an mRNA subset include, but are not limited to, differential display, phage display/analysis, SAGE (Serial Analysis of Gene Expression), and preparation of cDNA libraries from the mRNA preparation and sequencing of the members of the library.

Methods for DNA sequencing that are well known and generally available in the art may be used to practice any of the embodiments of the invention. The sequencing methods may employ such enzymes as the Klenow fragment of DNA polymerase I, SEQUENASE® (U.S. Biochemical Corp, Cleveland, OH), Taq polymerase (Perkin Elmer, Boston, MA), thermostable T7 polymerase (Amersham, Chicago, IL), or combinations of polymerases and proofreading exonucleases such as those found in the Elongase® Amplification System marketed by Gibco BRL (Invitrogen™, Carlsbad, CA). Preferably, the process is automated with machines such as the Hamilton Micro Lab 2200 (Hamilton, Reno, NV), Peltier Thermal Cycler (PTC200) (MJ Research, Watertown, MA) and the ABI Catalyst and 373 and 377 DNA Sequencers (Perkin Elmer, Shelton, CT).

In an embodiment, the methods of the invention are carried on isolated nuclei from cells (*e.g.*, that are undergoing developmental or cell cycle changes or that have otherwise been subjected to a cellular or an environmental change), performing nuclear run-off assays according to known techniques to obtain transcribing mRNAs, and then comparing the transcribing mRNAs with the global mRNA levels isolated from mRNP complexes from the same cells using cDNA microarrays. These methods thus provide methods for distinguishing transcriptional from post-transcriptional effects on steady state mRNA levels *en masse*. As opposed to a total RNA or transcription profile that depicts RNA accumulation representing a steady-state level of mRNA, which is affected by transcriptional and post-transcriptional events, the mRNAs detected by nuclear run-off experiments represent only the transcription of a gene before the influence of post-transcriptional events. The microarrays representing mRNP complexes contain discrete and more limited subsets of mRNAs than the transcriptome or nuclear run-offs. The mRNP complex microarrays are labeled mRNP-1 through mRNP-X and depict multiple mRNAs found in mRNP complexes isolated by using antibodies reactive with mRNP complex-associated proteins.

Other methods for characterizing and identifying mRNP complex components include standard laboratory techniques such as, but not limited to, reverse transcription or quantitative PCR, RNAse protection, Northern Blot analysis, Western blot analysis, macro- or micro-array analysis, *in situ* hybridization, immunofluorescence, radioimmunoassay, and immunoprecipitation. The results obtained from these methods are compared and contrasted in

order to characterize further the functional relationships of the mRNA subsets and other mRNP components.

RNA binding proteins and mRNP complex-associated proteins useful in the practice of the present invention are known in the art, or may alternatively be identified and discovered by the methods described herein. RNA binding proteins are involved in the control of a variety of cellular regulatory and developmental processes, such as RNA processing and compartmentalization, mRNA splicing and transport, RNA stabilization, mRNA translation, and viral gene expression. Examples of useful RNA binding proteins are shown in Table 1.

Table 1 RNA Binding Proteins Associated with Glucose Metabolism

RPP30	NCOR2	SNRPG	LOC165271
NCOA5	IMP-1	NFKB2	OAS1
DDX28	LOC56902	HNRD	RNASE2
RBMY1A1	GRTH	LOC163412P	RNF17
LOC136197	LOC168400	ENPP2	HE3-BETA
RRP46	PRIM2A	RPS15A	LOC129715
ZNF85	EIF-2Bepsilon	LOC126205	LOC127164
LOC134477	BOLL	HYPA	RPS21
PCBD	LOC151613	LOC138280	OAS3
YBX2	PSP1	HQK	PCBP3
A2BP1	FLJ12455	DDX21	

The techniques described herein are used to identify new (*i.e.*, novel or previously unknown) RNA binding proteins and mRNP complex-associated proteins associated with glucose metabolism is referred to as RiboTrap™ (Figure 4). Thus, in one embodiment of the invention, an mRNA of interest (depicted in Figure 4 as "RNA Y") is used as "bait" to trap a new RNA binding protein. Preferably, RNA Y is first converted to a cDNA using standard molecular biology techniques and is subsequently ligated at the 3' or 5' end to a DNA tag that encodes a sequence that will bind a ligand of the present invention (the ligand being illustrated as protein "X" in Figure 3). In other words, the tagged DNA encodes a binding partner of the ligand. The resulting fusion RNA is expressed in cells, where endogenous RNA binding proteins can bind and interact with RNA Y. The cells are then lysed and cell-free extracts are prepared and contacted with Protein X, which has been immobilized on a solid support matrix. After incubation, Protein X and the attached RNA fusion molecule and its associated RNA binding proteins are washed to remove residual cellular material. After washing, the newly isolated RNA binding proteins are removed from the RNA-protein complex and identified by protein microsequencing. Useful ligands include mRNP complex-specific antibodies or proteins (*e.g.*, obtained from a subject with an autoimmune disorder or cancer) or proteins. Useful binding partners include antibodies specific for the ligand.

Once partial protein sequence is obtained, the corresponding RNA binding protein gene may be identified from known databases of cDNA and genomic sequences or isolated from a cDNA or genomic library and sequenced. Preferably, the gene is isolated, the protein is expressed, and an antibody is generated against the recombinant RNA binding protein using known techniques. The antibodies are then used to recover and confirm the identity of the endogenous RNA binding protein. Subsequently, the antibody can be used for ribonomic analysis to determine the subset of cellular RNAs that cluster with (*i.e.*, associate with) RNA Y. The RNA binding protein is further tested for its ability to regulate the translation of the protein encoded by RNA Y, and is tested for validation as a drug target. Likewise, proteins encoded by the cellular RNAs that cluster with RNA Y are tested for validation as drug targets, as further described herein.

Identification of Therapeutic Targets

The invention provides methods for identifying a therapeutic target by comparing the ribonomic subprofiles of a cell sample to the ribonomic subprofiles of a control sample. A difference in the expression of a component of an mRNP complex between the two samples is indicative that the component is a candidate therapeutic target. The therapeutic target may include, but is not limited to, any component of an mRNP complex, or nucleic acid or gene product thereof. In an embodiment of the invention, the cell sample is treated with a test compound and the control sample comprises cells that have not been treated with the test compound. In another embodiment, the control sample comprises cells at a different stage in their growth cycle from the cells in the cell sample. In yet another embodiment, the cell sample comprises a tumor cell or other diseased cell, and the control sample comprises a normal cell. Target identification includes methods known to practitioners in the art, such as, but not limited to, the use of screening libraries, peptide phage display, cDNA microchip array screening, and combinatorial chemistry techniques known to practitioners in the art. A summary of the steps for target discovery is provided in Figure 5.

Identification of Therapeutics

In another aspect, the invention provides methods for assessing the efficacy of a test compound as a therapeutic. A cell sample is contacted with a test compound and a ribonomic profile or subprofile of the cell sample comprising the expression of at least one gene product associated with at least one mRNP complex is prepared. The expression levels of the gene product in the cell sample are compared to the expression levels of the gene product in a control sample (*e.g.*, a cell sample that is not contacted with a test compound). Identification of a difference in expression of the gene product between the treated and untreated cell samples is indicative that the test compound is a potential therapeutic. Test compounds may be, for example, nucleic acids, hormones, antibodies, antibody fragments, antigens, cytokines, growth factors, pharmacological agents (*e.g.*, chemotherapeutics, carcinogenics, or other cells), chemical compositions, proteins, peptides, and/or small molecules.

In various embodiments of the invention, the therapeutic may stabilize or destabilize the mRNA or the mRNP complex-associated protein. In another embodiment, the therapeutic may either inhibit translation of the mRNA, inhibit transport of the mRNA or the mRNP complex-associated protein, inhibit the binding of the RNA binding protein to the mRNA, inhibit the binding of the RNA binding protein to the mRNP complex-associated protein, or inhibit the binding of the mRNA to the mRNP complex-associated protein, for example.

In another aspect, the invention provides methods for assessing toxicity, potential side effects, specificity or selectivity of a test compound, for example, by altering the concentrations or amounts of a test compound used to treat a cell sample.

In yet another aspect, the present invention provides methods for assessing or monitoring the efficacy of a therapeutic in a subject. In accordance with the invention, an effective amount of a therapeutic is administered to a subject. At least one mRNP complex is isolated from a cell sample from the subject, wherein altered expression of a gene product associated with the mRNP complex is altered by administration of the therapeutic. The expression of the gene product in the cell sample after administration of the therapeutic is compared to the expression of the gene product in a control sample (*e.g.*, a second cell sample obtained either prior to administration of the therapeutic or from a normal subject). A difference in expression between the treated and the control cell samples is indicative of the efficacy of the therapeutic. The above tests can be repeated over a period of time to monitor the continued efficacy of the therapeutic.

Therapeutics may target over- or under-expressed proteins involved in the etiology of a disease, disorder, or condition. Such over- or under-expression may result in destabilization or stabilization of RNA.

Therapeutics that Destabilize mRNA

If a disease, condition or disorder is characterized by overexpression of a protein, a therapeutic for treatment of such a condition will reduce or eliminate expression of the protein. For example, since RNA binding proteins enhance the stability of short-lived mRNAs encoding protooncogenes, growth factors and cytokines that contribute to cell proliferation, inhibition of

RNA binding protein production may alleviate diseases such as cancers or autoimmune diseases (e.g., by decreasing tumor growth or inflammation, respectively). In addition, RNA binding protein overexpression in several human tumors correlates with resistance to chemotherapy and UV irradiation. Increased stability of c-fos, c-myc, cyclin B1 and other short-lived mRNAs in response to UV-irradiation or therapeutic drugs is well known. Accordingly, inhibition of RNA binding protein expression in these tumors destabilizes the mRNA in the tumors and, as a result, renders the tumors more responsive to cancer treatments.

In order to reduce overexpression or to cease expression of a protein of interest, the mRNA can be destabilized by administering an effective amount of a suitable test compound (*e.g.*, an RNA binding protein inhibitor) either *in vitro* or *in vivo*. The test compound may bind mRNA so as to inhibit RNA binding protein binding to the mRNA, bind the RNA binding protein so as to inhibit RNA binding protein binding to the mRNA, bind to and destabilize the mRNP complex, and/or bind the mRNA so as to directly destabilize the mRNA, for example. Compounds that bind to the mRNA but that do not stabilize the mRNA may inhibit the ability of an RNA binding protein to stabilize the mRNA. If the compound binds competitively with an RNA binding protein, the compound can decrease mRNA stability by inhibiting the RNA binding protein's ability to bind the mRNA.

Alternatively, the test compound may inhibit RNA binding protein or mRNA expression.

Effective test compounds (*e.g.*, RNA binding protein inhibitors) can be readily determined by screening compounds for their ability to interfere with the production of RNA binding protein or their ability to inhibit the binding to, and/or stabilization of, mRNA, for example, by methods described herein. Compounds that function by inhibiting RNA binding protein or mRNA production can be identified by exposing cells that express the RNA binding protein or mRNA of interest and monitoring the levels of RNA binding protein or mRNA, respectively. Compounds that function by inhibiting the stabilizing effect of RNA binding protein on mRNA can be identified by combining RNA binding protein and an mRNA that would otherwise be stabilized, adding compounds to be evaluated as RNA binding protein inhibitors, and monitoring the binding affinity of RNA binding protein and the mRNA.

Compounds that increase or decrease the binding affinity of RNA binding protein and the mRNA can be readily determined by art known methods.

Therapeutics that Stabilize mRNA

If a disease, condition or disorder is characterized by underexpression of an mRNA stabilizing protein, a therapeutic for treatment of such a medical condition may operate by stabilizing the mRNA associated with the underexpressed protein. Accordingly, mRNA may be stabilized by administering an effective amount of a compound, either *in vitro* or *in vivo*. The compound may possess a similar binding ability and stabilizing effect as the RNA binding protein or, may promote the RNA binding protein's ability to stabilize mRNA, and/or may promote the production of the stabilizing RNA binding protein or the mRNA of interest. Such a compound may be referred to as an RNA binding protein inducer and may operate by interacting with the mRNA, the RNA binding protein or both. Alternatively, mRNA can be stabilized by administering an effective amount of a suitable RNA binding protein that possesses the necessary mRNA stabilizing effect.

Compounds that increase RNA binding protein production can be identified by initially exposing cells that express the RNA binding protein to potential inducers and, monitoring the levels of the RNA binding protein, in accordance with the methods described above. If the level of RNA binding protein expression increases, the compound is an RNA binding protein inducer.

Compounds that inhibit RNA binding protein binding to mRNA, but which bind and stabilize mRNA, can be identified by methods disclosed herein. A skilled practitioner may combine RNA binding protein and an mRNA that would otherwise be stabilized, add a compound, and monitor the binding affinity of the RNA binding protein and the mRNA. Compounds that increase or decrease the binding affinity of an RNA binding protein and the mRNA can be readily determined by evaluating the binding affinity of the RNA binding protein to the mRNA after exposure to the compound, as described herein. By monitoring the concentration of mRNA over time, those compounds that bind to the mRNA can then be assayed for their ability to stabilize mRNA.

Antibody Preparation

Antibodies and fragments thereof that bind to mRNP complexes are generated using methods that are well known in the art. Such antibodies may include, but are not limited to, polyclonal, monoclonal, chimeric, single chain, Fab fragments, and fragments produced by a Fab expression library. Antibodies and fragments thereof may also be generated using antibody phage expression display techniques, which are known in the art.

For the production of antibodies, various hosts including, but not limited to, goats, rabbits, rats, mice, and humans are immunized by injection with the mRNP complex or any fragment or component thereof that has immunogenic properties. Depending on the host species, an adjuvant is used to increase the immunological response. Such adjuvants include, but are not limited to, Freund's, mineral gels such as aluminum hydroxide, and surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, and dinitrophenol. Among adjuvants used in humans, BCG (Bacilli Calmette-Guerin) and Corynebacterium parvum are preferable.

Monoclonal antibodies to the components of the mRNP complex are prepared using any technique that provides for the production of antibody molecules by a cultured cell line. These include, but are not limited to, the hybridoma technique, the human B-cell hybridoma technique, and the EBV-hybridoma technique. Generally, an animal is immunized with the mRNP complex or immunogenic fragment(s) or conjugate(s) thereof. Lymphoid cells (*e.g.*, splenic lymphocytes) are then obtained from the immunized animal and fused with immortalized cells (*e.g.*, myeloma or heteromyeloma) to produce hybrid cells. The hybrid cells are screened to identify those that produce the desired antibody.

Antibodies may also be produced by inducing *in vivo* production in the lymphocyte population or by screening immunoglobulin libraries or panels of highly specific binding reagents as is known in the art.

Antibody fragments that contain specific binding sites for mRNP complexes may also be generated. For example, such fragments include, but are not limited to, the $F(ab')_2$ fragments

which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragments. Alternatively, Fab expression libraries are constructed to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

Various immunoassays are used to identify antibodies having the desired specificity for the mRNP complex. Numerous protocols for competitive binding or immunoradiometric assays using either polyclonal or monoclonal antibodies with established specificities are well known in the art. Such immunoassays typically involve the measurement of complex formation between the component of the mRNP complex and its specific antibody. An immunoassay utilizing monoclonal antibodies reactive to two non-interfering epitopes is preferred, but a competitive binding assay may also be employed.

The invention provides kits containing columns in which antibodies to various mRNP complexes or components thereof (*e.g.*, antibodies to RNA binding proteins) are immobilized. The antibodies may be conjugated to a support suitable for a diagnostic assay (*e.g.*, a solid support such as beads, plates, slides or wells formed from materials such as latex or polystyrene) in accordance with known techniques. Antibodies may likewise be conjugated to detectable groups such as radiolabels (*e.g.*, ^{35}S, ^{125}I, ^{131}I), enzyme labels (*e.g.*, horseradish peroxidase, alkaline phosphatase), and fluorescent labels (*e.g.*, fluorescein) in accordance with known techniques. Such devices preferably include at least one reagent specific for detecting the binding between an antibody and the RNA binding protein. The reagents may also include ancillary agents such as buffering agents and protein stabilizing agents (*e.g.*, polysaccharides and the like). The device may further include, where necessary, agents for reducing background interference in a test, control reagents, apparatus for conducting a test, and the like. The device may be packaged in any suitable manner, typically with all elements in a single container, along with a sheet of printed instructions for carrying out the test.

Antibodies raised against an mRNP complex can be conjugated to a drug. Upon administration to a patient, the antibody will bind to the mRNP complex so as to deliver a relatively high concentration of the drug to the desired tissue or organ. In one embodiment, an

antibody is conjugated to an anti-cancer drug, including, but not limited to, an antifolate, an anti-tumor antibiotic and other tumor-treating compound

Antibodies that bind to the mRNP complex can also be covalently or ionically coupled to various markers, and used to detect the presence of tumors. By administering a suitable amount of the marker-coupled antibody to a patient, allowing the antibody to bind the mRNP complex at or around a tumor site, and detecting the marker, the presence of tumors can be detected. Suitable markers are well known in the art, and include, but are not limited to, radioisotopic labels, fluorescent labels and the like. Suitable detection methods for these markers are also well known in the art and include, but are not limited to, positron emission tomography, autoradiography, flow cytometry, radioreceptor binding assays, and immunohistochemistry.

High Throughput Screening Methods for Libraries of Compounds

In an embodiment of the invention, high throughput screening assays and competitive binding assays are used to identify compounds that bind to an mRNP complex or component thereof from combinatorial libraries of compounds (*e.g.*, phage display peptide libraries, small molecule libraries and oligonucleotide libraries).

In one embodiment, an mRNP component, catalytic or immunogenic fragment thereof, or oligopeptide thereof, can be used to screen libraries of compounds in any of a variety of drug screening techniques. An exemplary technique is described in published PCT application W084/03584, hereby incorporated by reference. The fragment employed in such screening can be free in solution, affixed to a support (*e.g.*, solid support), borne on a cell surface, or located intracellularly.

The SELEX method, described in U.S. Patent No. 5,270,163 (Gold *et al.*), hereby incorporated by reference, is used to screen oligonucleotide libraries for compounds that have suitable binding properties. In accordance with the SELEX method, a candidate mixture of single stranded nucleic acids with regions of randomized sequence can be contacted with the mRNP complex. Those nucleic acids having an increased affinity to the mRNP complex can be partitioned and amplified so as to yield a ligand enriched mixture.

Phage display technology is used to screen peptide phage display libraries to identify peptides that bind to an mRNP complex or component thereof. Methods for preparing libraries containing diverse populations of various types of molecules such as peptides, polypeptides, proteins, and fragments thereof are known in the art and are commercially available.

A library of phage displaying potential binding peptides is incubated with an mRNP complex to select clones encoding recombinant peptides that specifically bind the mRNP complex or components thereof. After at least one round of biopanning (binding to the mRNP complex), the phage DNA is amplified and sequenced, thereby providing the sequence for the displayed binding peptides. Briefly, the target, an mRNP complex, can be coated overnight onto tissue culture plates and incubated in a humidified container. In a first round of panning, approximately 2×10^{11} phage can be incubated on the protein-coated plate for 60 minutes at room temperature while rocking gently. The plates are then washed using standard wash solutions. The binding phage can then be collected and amplified following elution using the target protein. Secondary and tertiary pannings can be performed as necessary. Following the last screening, individual colonies of phage-infected bacteria can be picked at random, the phage DNA isolated and subjected to automated dideoxy sequencing. The sequence of the displayed peptides can be deduced from the DNA sequence.

The biological activity of compounds can be evaluated using *in vitro* assays known to those skilled in the art (*e.g.*, protein synthesis assays or tumor cell proliferation assays). Alternatively, the biological activity of the compounds is evaluated *in vivo*. Various compounds, including antibodies, can bind to mRNP complexes and components thereof with varying effects on mRNA stability. The activity of the compounds once bound can be readily determined using the assays described herein.

Binding assays include cell-free assays in which an RNA binding protein and an mRNA are incubated with a labeled test compound. Following incubation, the mRNA, free or bound to a test compound, can be separated from unbound test compound using any of a variety of techniques known in the art. The amount of test compound bound to an mRNP complex or component thereof is then determined, using detection techniques known in the art.

Alternatively, the binding assay is a cell-free competition binding assay. In such assays, mRNA is incubated with labeled RNA binding protein. A test compound is added to the reaction and assayed for its ability to compete with the RNA binding protein for binding to the mRNA. Free labeled RNA binding protein can be separated from bound RNA binding protein. By subsequently determining the amount of bound RNA binding protein, the ability of the test compound to compete for mRNA binding can be assessed. This assay can be formatted to facilitate screening of large numbers of test compounds by linking the RNA binding protein or the mRNA to a support so that it can be readily washed free of unbound reactants. A plastic support (*e.g.*, a plastic plate such as a 96 well dish) is preferred. The RNA binding protein and mRNA suitable for use in the cell-free assays described herein can be isolated from natural sources (*e.g.*, membrane preparations) or prepared recombinantly or chemically. The RNA binding protein can be prepared as a fusion protein using, for example, known recombinant techniques. Preferred fusion proteins include, but are not limited to, a glutathione-S-transferase (GST) moiety, a green fluorescent protein (GFP) moiety that is useful for cellular localization studies or a His tag that is useful for affinity purification.

A competitive binding assay may also be cell-based. Accordingly, a compound, preferably labeled, known to bind an mRNP complex or component thereof, is incubated with the mRNP complex or component thereof in the presence and absence of a test compound. By comparing the amount of known test compound associated with cells incubated in the presence of the test compound with that of cells incubated in the absence of the test compound, the affinity of the test compound for the RNA binding protein, mRNA, and/or complex thereof can be determined. Cell proliferation can be monitored by measuring the uptake into cellular nucleic acids of labeled bases (*e.g.*, radioactively, such as ^{3}H, SiC, or ^{14}C; fluorescently, such as CYQUANT (Molecular Probes); or colorimetrically such as rdU (Boehringer Mannheim) or MTS (Promega)) as known in the art. Cytosolic/cytoplasmic pH determinations can be made with a digital imaging microscope using substrates such as bis(carboxyethyl)-carbonyl fluorescein (BCECF) (Molecular Probes, Inc., Eugene, Oregon).

Other types of assays that can be carried out to determine the effect of a test compound on RNA binding protein binding to mRNA include, but are not limited to, the Lewis Lung Carcinoma assay and extracellular migration assays such as the Boyden Chamber assay.

Accordingly, the methods permit the screening of compounds for their ability to modulate the effect of an RNA binding protein on the binding of and stability of mRNA. Using the assays described herein, compounds capable of binding to mRNA and modulating the effects on those cellular bioactivities resulting from mRNA stability and correlated protein synthesis are identified. The compounds identified in accordance with the above assays are formulated as therapeutic compositions.

Diagnosing and Monitoring Disease

In another aspect, the invention provides methods for diagnosing a disease or risk of a disease related to glucose metabolism (e.g., obesity or diabetes) or cellular function. A ribonomic profile from a subject's cell sample is prepared and at least one mRNP complex is analyzed. The expression of at least one gene product, for which altered expression is indicative of a disease or risk of disease, is determined. The gene product may be an RNA binding protein, an mRNA, an mRNP complex-associated protein or other gene product bound to or associated with the mRNP complex. The expression of the gene product in the cell sample is compared to the expression of the gene product in a control sample. The control sample may be either a sample of normal cells or a second cell sample from the subject. Alternatively, the control sample is a positive control from a diseased and/or normal individual. By observing the relative expression of the gene product in the cell sample compared to the control sample, the presence of a disease or risk of disease can be determined.

In another aspect, the invention discloses a method for monitoring a disease state in a subject. At least one mRNP complex is isolated from a diseased subject's cell sample, wherein the mRNP complex has at least one gene product that is associated with the disease. The expression of the gene product in the subject's cell sample is compared to the expression of the gene product in a control sample. The identification of a difference in the expression of the gene

product in the diseased subject cell sample compared to the expression of the gene product in the control sample is indicative of a change in the disease state of the subject. For example, a decrease in the production of a tumor related antigen or its mRNA is indicative of decreased tumor load or remission; by contrast, an increase in expression of the tumor antigen is indicative of aggressive tumor growth. Such monitoring during drug treatment provides information about the effectiveness of the subject's drug regimen, and may indicate when a particular regimen is not, or is no longer, effective for treating the disease or condition. The control sample may be a second cell sample from the subject, preferably, obtained when the subject is free of one or more symptoms of the disease. Alternatively, the control sample is from a normal subject or other normal cell sample.

In summary, the present invention provides powerful *in vivo* methods for determining the ribonomic profile of a cell and detecting changes in the ribonomic profile. The invention has numerous uses, including, but not limited to, monitoring cell development or growth, monitoring a cell state, and monitoring perturbations of a biological system such as disease, condition or disorder. The invention further provides methods for diagnosing a disease, condition, or disorder and determining appropriate treatment regimens. The invention also is useful for distinguishing ribonomic profiles among organisms such as plant, fungal, bacterial, viral, protozoan, or animal species.

The present invention can be used to discriminate between transcriptional and post-transcriptional contributions to gene expression and to track the movement of RNAs through mRNP complexes, including the interactions of combinations of proteins with RNAs in mRNP complexes. Accordingly, the present invention can be used to study the regulation of RNA stability. The present invention can be used to investigate the activation of translation of mRNAs as single or multiple species by tracking the recruitment of mRNAs to active polysomes, measuring the sequential, ordered expression of mRNAs such as mRNAs that encode transcription factors or RNA binding proteins, and measuring the simultaneous, coordinate expression of multiple mRNAs. The present invention can also be used to determine the transacting functions of RNAs themselves upon contacting other cellular components. These

and numerous other uses will be made apparent to the skilled artisan upon study of the present specification and claims.

The following Examples are set forth to illustrate the present invention, and are not to be construed as limiting thereof.

Exemplification

Example 1: Target Discovery Using Ribonomic Profiles

The general steps required for target discovery using the methods of the invention are summarized in Figure 1 and are exemplified below.

Establishing Expression Profiles for RNA Binding Protein Genes

RNA binding protein expression profiles are generated in normal and diseased human tissues or in tissues that are treated with an agent, such as glucose, an inhibitor, and/or insulin. Initial tissue, disease, or agent screening of RNA binding proteins is accomplished by Quantitative Reverse Transcriptase-PCR using oligo dT-primers and commercially available RNA samples (Stratagene, Inc., La Jolla, CA; Ambion, Inc., Austin, TX; BD Biosciences Clontech, Palo Alto, CA). 10-100μg of cDNA is used to perform Quantitative PCR using SybrGreen (Molecular Probes, Inc., Eugene, OR) and gene specific PCR primers on a BioRad iCycler Quantitative PCR machine (Biorad) using protocols provided by the manufacturer. Experimental results are analyzed using the accompanying BioRad iCycler software. RNA levels for candidate RNA binding protein genes are normalized to rRNA.

For more rapid and comprehensive screening of tissues and cell lines, a custom RIBOCHIP™ spotted microarray (Ribonomics, Inc., Durham, NC), designed and manufactured under contract by MWG Biotech USA, Highpoint, NC) may be used. The Ribochip™ contains known and putative human RNA binding protein genes, compiled from a wide variety of public databases and search tools including GenBank (NCBI, Bethesda, MD), PubMed (NCBI, Bethesda, MD), SRS Evolution (LION Biosciences, Cambridge, MA), LocusLink (NCBI, Bethesda, MD), Protein FAMily database (pFAM); Welcome Institute Sanger Institute (Hinxton,

UK), GO Database (Gene Ontology™ Consortium) and Structural Classification of Proteins (SCOP©) Package (Medical Research Council, Cambridge, UK). This array contains 50-mer oligonucleotides on glass slides corresponding to greater than about 1,400 RNA binding protein genes in duplicate, non-contiguous positions (plus control genes).

To screen for the expression of RNA binding proteins, RNA is prepared from cells in culture and from snap frozen clinical tissues according to the Qiagen RNeasy® protocol (Qiagen, Inc., Valencia, CA), quantified by absorbance at A_{260}, and stored at -80°C until use. RNA samples are routinely evaluated using an AGILENT Bioanalyzer 2100, which provides a rapid and quantitative measure of 28S AND 18S ribosomal RNA integrity. Total or poly A+ RNA is labeled without amplification through generation of cDNA by reverse transcription in the presence of amino allyl-dUTP followed by direct coupling to Cy3 or Cy5 fluorescent dyes (TIGR SOP#M0004). The labeled RNA is used to probe the Ribochip™ to identify and/or quantify the level of RNA binding protein mRNA in the sample. Hybridization and washing are performed by standard procedures (TIGR SOP#M0005). If RNA quantities are limiting (< 10 μg), nucleic acid amplification is accomplished with reverse transcription, cDNA tailing, and PCR amplification procedures prior to Cy3/Cy5 coupling. Data flow is then analyzed as summarized in Figure 3. Briefly, the Ribochip™ microarray slides are first scanned and read by a GENEPIX® Axon 4000B scanner using GENEPIX® 4.0 software (Axon Instruments, Inc., Union City, CA) for data acquisition. Spot features are then extracted with Biodiscovery's IMAGENE™ V.4.2 package (BioDiscovery, Inc., Marina Del Rey, CA). Data preprocessing, including intra- and inter-array data normalization, centralization, and scaling, is accomplished by visual (*e.g.*, heat map) and quantitative (*e.g.*, distribution analysis) methods implemented using the statistical environment R (Ross Ihaka and Robert Gentleman; R: A language for Data Analysis and Graphics, Journal of Computational and Graphical Statistics, 1996, 5, 299-314; hereby incorporated by reference) and BioConductor Suite of microarray data normalization and analysis libraries (BioConductor, Biostatistics Unit of Dana Farber Cancer Institute, Boston, MA). Final data analysis with normalization and scaling is then accomplished using gene clustering, statistical filtering and class prediction functions within the GENESPRING® 4.2.1 software platform (Silicon Genetics, Redwood City, CA). Differentially expressed genes are

identified using a combination of methods for assessing variation in expression level between conditions. These Include t-tests and ANOVA analysis with multiple testing corrections, fold difference filtering, and clustering techniques. These methods enable genes with unique or "interesting" expression profiles to be quickly identified from among the thousand genes profiled in a single experiment. Based upon array data, RNA binding proteins that are up or down regulated (*e.g.*, differential RNA binding protein mRNA levels) to a statistically significant extent in a tissue-, disease-, or agent-specific manner are selected for confirmation studies by Quantitative PCR, Northern blot analysis, Western blot analysis, for example.

To evaluate sample to sample variability, a series of self-self comparisons are performed. Using identical RNA samples, procedural variability in spot intensity is examined as a function of different slides, print tips, label, days, and investigators. Raw intensities for each of 1400 spots on a custom array are compared following labeling with Cy3 or Cy5 fluorescent dyes. Based upon these analyses, the pooled coefficient of variation for between slide comparisons is determined (e.g., 11%). This represents an extremely high level of reproducibility for microarray analysis of gene expression.

Cloning and Expression of RNA Binding Protein Genes in Bacterial Vectors

As soon as candidate, differentially expressed RNA binding proteins are identified, full length cDNA clones are generated by reverse transcriptase-PCR using commercial RNA tissue sources. Full-length plasmid clones are constructed based on phage lambda-based (att) site-specific recombination protocols (Invitrogen, Corp., Carlsbad, CA) for the GATEWAY™ pENTRD-Topo entry vectors and pDEST17 6XHis destination vectors (Invitrogen, Corp., Carslbad, CA). *Escherichia coli* (*e.g.*, BL21SI or BL21A1) expressing polyhistidine-tagged RNA Binding Protein fusion proteins are grown to mid-log phase at 37 °C and induced in 0.3 M NaCl for BL21SI cells or in 0.2% mM arabinose or 0.1mM IPTG for BL21A1 cells at 20-37 °C for about 2-6 hours (specific time based upon optimization in pilot expression studies for each clone). Bacterial cells are lysed by sonication and the fusion protein is purified on nickel columns (Qiagen, Inc., Valencia, CA) using standard methods. Insoluble fusion proteins are maintained and purified in the presence of 8M urea, and soluble proteins are maintained in PBS. Purified recombinant proteins are used for immunization of mammals (e.g., rabbits or chickens)

for production of polyclonal antibodies using standard methods or produced through a commercial contract. Polyclonal antibodies are characterized by their ability to immunoprecipitate and detect by western blot, for example, native and recombinant proteins.

Interrogation of mRNP complexes

RNA binding proteins that are expressed in a tissue-, disease-, or agent-specific manner are markers for the cellular alterations that occur in response to the post-transcriptional processing of functionally related mRNAs. Changes in the abundance or constellation of RNA binding proteins in a cell affects the processing of any mRNAs bound to those RNA binding proteins. The subset of mRNAs that are associated with an RNA binding protein is indicative of functional co-regulation that is critically or causally involved in effecting a phenotype change in the cell. Thus, as a subset, those genes whose mRNAs are associated with tissue-, disease-, or agent-specifically altered mRNP complexes are a rich source of therapeutic targets for drug discovery.

RNA binding proteins that exhibit the most dramatic variation with regard to expression proceed into the second stage of analysis, the Ribonomic Analysis System (RAS™) assay (Ribonomics, Durham, NC) a flow chart for which is illustrated in Figures 1A and 1B. The RAS™ assay provides affinity isolation and characterization of *in vivo* formed the mRNA complexes containing mRNA, RNA binding proteins, and/or mRNP complex-associated proteins. Monoclonal or polyclonal antibodies raised to a specific RNA binding protein, mRNP complex-associated protein, or a tag on an RNA binding protein or mRNP complex-associated protein of interest are used to co-immunoprecipitate and isolate the RNA binding protein and the associated subset of mRNAs and mRNP complex associated proteins according to standard methods or the manufacturers instructions. The associated RNAs are extracted and analyzed in a standard microarray format to identify and/or quantify the specific mRNAs associated with the RNA binding proteins.

For the analysis of mRNAs associated with mRNP complexes, commercially available glass slide arrays (such as, e.g., Agilent Human Unigene 14K (Agilent, Palo Alto, CA) and

MWG Pan Human 10K (MWG Biotech, Inc., High Point, NC), or membrane arrays, such as, for example, Atlas™ Arrays (BD Biosciences, Clontech, Palo Alto, CA)), may be employed using protocols for hybridization, washing, and development provided by the array manufacturers.

The composition of RAS™ assay lysis buffer (RLB) may vary, depending on the binding characteristics of a particular RNA binding protein. Basic RLB contains 50 mM HEPES, pH 7-7.4, 1% NP-40, 150 mM NaCl, 1 mM DTT, 100 U/ml RNase OUT, 0.2 mM PMSF, 1 μg/ml aprotinin and 1 ug/ml leupeptin. Variations of these basic components included changes in salt concentrations (e.g., about 0-500 mM NaCl or about 0-5 mM KCl), ionic conditions (about 0-10 mM $MgCl_2$ or about 0-20 mM EDTA), and reducing environment (about 0-5 mM DTT). For example, in order to prepare cell extracts for examining the polypyrimidine tract binding protein (PTB) mRNP, cultured cells are washed in ice-cold PBS and scraped directly into RLB containing 5 mM $MgCl_2$ and incubated on ice for 10 minutes followed by centrifugation at 3,700 xg for 10 minutes at 4 °C.

It is necessary in certain cases to crosslink the RNA binding protein to target mRNAs prior to lysis and mRNP isolation. This is performed on cultured cells as well as fresh tissue samples. The extent of crosslinking is titrated for each cell line or tissue and monitored based on ability to immunoprecipitate mRNA in the complex. Cultured cells or tissue are incubated in phosphate buffered saline (PBS) containing about 0-1% formaldehyde at room temperature for about 15 - 60 minutes. Crosslinking is then quenched by the addition of 1M Tris to a final concentration of 250 mM and incubated further for an additional 20 minutes. The samples are then washed 3x in PBS containing 50 mM Tris. For cultured cells, the pellet is resuspended in radioimmunoprecipitation (RIPA) buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% DOC and 100 U/ml RNase Out) at approximately 2 mg/ml final protein concentration. For tissue, the samples are resuspended in RIPA and homogenized with a Polytron to disrupt the tissue. Following the initial lysis, the samples (e.g., tissue or cultured cells) are subjected to sonication with a probe sonicator at output setting 6 (Branson 450, Branson Ultrasonics Corp., Danbury, CT) two times for 20 seconds each. Between sonications the samples are allowed to cool on ice for 2 minutes. Lysates are then cleared by centrifugation at 3,700 x g for 15 minutes. Subsequently, mRNP isolation as described above is performed.

Immunoprecipitation of mRNP and RNA Extraction

On average, typical final protein concentrations for the cellular lysates are 2 mg/ml. Approximately 2mg protein is used for each immunoprecipitation condition. The cleared cellular extracts are incubated with primary antibody (*e.g.*, anti-PTB is used at a final concentration of 10 µg/ml) or a control antibody at equal concentration (*e.g.*, pre-immune or IgG sera at final concentration of 10 µg/ml) for 2 hours at 4 °C. A 25 µl aliquot of Protein A Trisacryl (Pierce Biotechnology, Rockford, IL) is added and the samples rotated for 1 hour at 4°C. The immune complex is then washed 6x in RLB buffer by adding 1ml/wash of RLB buffer followed by brief centrifugations in a microcentrifuge for 30 seconds at 5,000 rpm. After the final wash, 50 µl of RNA extraction buffer from the PicoPure™ RNA isolation kit (Arcturus, Inc., Mountain View, CA) is added to the beads, vortexed briefly and centrifuged to pellet the beads. The extracted RNA is purified following the PicoPure™ protocol (Arcturus, Inc., Mountain View, CA). RNA present in the mRNP complex is then quantified using the RiboGreen™ assay (Molecular Probes, Inc., Eugene, OR).

Amplification of RNA for Microarray Analysis

Since mRNA isolated from mRNP complexes represents only a small subset of total RNA, isolated mRNA may be amplified prior to labeling. Message Amp™ (Ambion, Inc., Austin, TX) is used for RNA amplification according to the manufacturer's instructions. Two rounds of amplification are performed prior to labeling by random primer polymerization with Cy3 or Cy5-dUTP. Hybridization and washing are performed according to the microarray manufacturer's protocols and as described above. Microarray data acquisition and analysis are performed as described above.

Analysis of mRNP Cluster Microarray Results

In a standard RAS™ analysis (*e.g.*, normal vs. disease cells, treated vs. untreated cells), quantitative and qualitative changes in the total RNA content are compared to changes in the mRNP complex. The data obtained is fractionated into four classes: (1) mRNAs that show comparable quantitative changes in the mRNP complex, (2) RNAs present in the total RNA but

not in the mRNP complex, (3) RNAs present in the mRNP complex but apparently absent or below the level of detection in total RNA, and (4) RNAs that change in the cluster in a quantitatively different manner than in the total RNA analysis. In addition, the RAS™ assay identifies genes represented by class 4 that do not change in total abundance but that are repartitioned within the cell for alternative processing and regulation. As a result, different splice variants may be translated, the mRNA might be transported to and translated at a specific location within the cell, or translation itself might be up or down modulated. The subsets of genes identified within groups 3 and 4 cannot readily be identified by any other currently available approach to characterization of gene expression. Analysis of mRNP complexes reveals mRNAs that are enriched in the complex but otherwise present at sufficiently low levels to be lost to background in the total RNA.

The methods of the invention identify genes that participate in the cellular pathways that contribute to the phenotypic changes associated with disease or certain cellular states and thus are attractive targets for drug discovery. In addition, the methods of the invention identify target classes that have proven to be tractable targets for small molecule drugs. These target classes include nuclear receptors (e.g., hormone receptors), G-protein coupled receptors, phosphodiesterases, kinases, proteases, and ion channels, among others. Other target classes of therapeutic interest include secreted molecules, extracellular ligands, and phosphatases. Among these gene classes, particularly attractive targets are those with the most restricted systemic expression profile.

The RNA binding proteins identified as having altered expression in response to treatments, disease, or cell cycle changes are useful for targeting upstream signaling molecules for regulation (e.g., kinases). In addition, RNA binding proteins may be candidates for gene therapy (i.e., gene replacement) or therapeutic antibody targets.

Functional Verification of mRNP Complexes

For candidate target genes or gene products identified by the RAS™ assay, expression at the RNA and protein levels is confirmed by quantitative PCR and Western blot. Furthermore,

the function of an mRNP complex as it relates to the fate of the associated mRNAs, such as stability, degradation, or subcellular localization, is explored through a variety of techniques including, but not limited to, confocal microscopy, *in situ* hybridization, 3-hybrid reporter analysis to confirm ternary interaction between mRNA and the RNA binding protein or mRNP complex-associated protein, and *in vitro* methods assessing biochemical activities (e.g., overexpression or knockout studies). Such studies are supported by the *in vitro* demonstration of RNA binding protein binding to specific nucleotide sequences typically found in the 5' or 3' untranslated regions of the mRNA.

Example 2: Identification and Immunoprecipitation of Preproinsulin RNA Binding Proteins Using RiboTrap™

An alternative method for purifying and identifying RNA binding proteins is the RiboTrap™ assay (Ribonomics, Durham, NC). Two approaches for RiboTrap™ are described below. The first approach is an *in vitro* affinity-based assay using immobilized biotinylated oligonucleotides with sequences corresponding to RNA binding protein binding elements (Figure 4). The second approach uses an affinity-tag placed on a full-length mRNA of interest or fragment of the mRNA of interest, which is expressed in a cell culture model and isolated using immobilized antibodies against the tag (Figure 6).

To summarize, a cDNA representing a gene of interest or a 5' and/or 3' UTR, or other region of the cDNA that encodes an RNA binding protein binding site, is constructed using standard recombinant DNA or PCR techniques. For example, the cDNA is cloned into an expression vector possessing an appropriate mammalian cell promoter such as a CMV, SV40 or actin promoters, or alternatively an adenovirus or retrovirus vector, and transfected into a compatible mammalian cell line. For example, for the isolation of RNA binding proteins that participate in glucose metabolism, the cDNA may be expressed in a preadipocyte, adipocyte, or pancreatic beta cell line, for example. Following expression of the engineered cDNA, a cell extract is prepared that maintains the association between mRNAs and their associated RNA binding proteins and mRNP complex-associated proteins as described above. The mRNA encoded by the transfected cDNA is affinity purified using an affinity protein that is known to

bind to the mRNA of interest, preferably one that does not interfere with the binding of the mRNA to its RNA binding protein(s) (Method 1 below; Figure 4). The affinity protein used may be linked to a solid matrix, such as agarose or Sepharose beads and may be biotinylated or otherwise labeled. The affinity protein may also be bound to the solid matrix indirectly via binding to an antibody that is bound to the solid matrix (Method 2 below; Figure 6). The affinity protein-matrix is used to isolate the expressed RNA, along with the RNA binding proteins and/or mRNP complex-associated proteins that are associated with the mRNA *in vivo*. Variations on the method include chemical crosslinking of the RNA binding proteins and mRNAs with formaldehyde or the use of an epitope tagged or beaded binding element or an epitope tagged mRNA of interest.

Proteins that are isolated in association with the mRNA of interest using the RiboTrap™ assay can be identified using standard proteomic methods. For example, Matrix Assisted Laser Desorption/Ionization - Time-of-Flight Mass Spectrometry (MALDI TOF) and Tandem Mass Spectrometry (or Mass Spectrometry/Mass Spectrometry (MS/MS)) can be used to identify peptide sequences that can be subjected to database searches. Antibodies reactive with identified RNA binding proteins or mRNP complex-associated proteins can be raised in mammals according to standard methods and used to perform the RAS™ assay, as described previously.

Methods and Materials

Preparation of Cell Free Extracts

Cells were removed from tissue culture plates with a rubber scraper and washed with cold phosphate buffered saline (PBS). The cells were resuspended in approximately two pellet volumes of polysome lysis buffer (PLB) containing 100 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES pH 7.0, and 0.5 % NP-40 with 1 mM Dithiothrietol (DTT), 100 U/mL RNase OUT (Gibco BRL, Invitrogen Corp., Carlsbad, CA), 0.2 % vanadyl ribonucleoside complex (VRC) (Gibco BRL, Invitrogen Corp., Carlsbad, CA), 0.2 mM Phenylmethylsulfonylfluoride (PMSF), 1 mg/mL pepstatin A, 5 mg/mL pepstatin, and 20 mg/mL leupeptin added fresh at the time of use. The cell lysate was frozen and stored at –100 °C. At the time of use, the cell lysate was thawed and centrifuged at 12,000 rpm in a tabletop microfuge for 10 minutes at 4°C. The supernatant was

removed and centrifuged a second time at 16,000 rpm in a tabletop microfuge for 5 minutes at 4°C before being stored on ice or refrozen at -100°C. The mRNP containing cell lysate contained approximately 30-50 mg/mL total protein.

Immunoprecipitation

Protein A sepharose beads (Sigma Biochemicals, St. Louis, MO) were swollen 1:5 v/v in NT2 buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, and 0.05 % NP-40) and supplemented with 5.% BSA A 300 µL aliquot of the 1:5 v/v pre-swollen. Protein A beads were incubated overnight at 4 °C with excess immunoprecipitation antibody (typically 5-20 µL, depending on the antibody). The antibody-coated Protein A beads were washed 5 times with ice-cold NT2 buffer and resuspended in 900 µL of NT2 buffer supplemented with 100 U/mL RNase OUT, 0.2 % Vanady/Ribonucleoside Complexes, 1 mM DTT, and 20 mM ethylene diaminetetracetic acid (EDTA). The beads were briefly vortexed and 100 µL of the mRNP lysate was added. The beads were immediately centrifuged and a 100 µL aliquot was removed to represent total cellular RNA (essentially one-tenth the quantity of lysate used in the mRNP immunoprecipitations). The immunoprecipitation reaction and an aliquot removed to represent total cellular RNA were tumbled at room temperature for a time period of from zero minutes to about two hours. Following an appropriate incubation, the Protein A beads were washed four times with ice-cold NT2 buffer followed by two washes with NT2 buffer supplemented with 1 M urea. The washed beads were resuspended in 100 µL NT2 buffer supplemented with 0.1 % sodium dodecyl sulphate (SDS) and 30 µg proteinase K and incubated for 30 minutes in a 55°C water bath. Following proteinase K digestion, immunoprecipitated RNA was isolated with two phenol/chloroform/isoamyl alcohol extractions and ethanol precipitated.

Method 1: *In Vitro* Affinity-Based Assay Using Immobilized Biotinylated Oligonucleotides

An *in vitro* affinity-based assay using immobilized biotinylated oligonucleotides is shown in Figure 4. Probes for affinity-purification of preproinsulin RNA binding proteins were synthesized and biotinylated with biotin-modified T (thymidine) by art known methods (e.g., Ross et al. (1997) Mol. Cell. Biol. 17:2158-65). The probes for purification of preproinsulin

RNA binding proteins were the following: a) for 3'-UTR element one 5'-gaauaaaaccuuugaaagagcacuac-3', b) for 3'-UTR element two 5'-cccaccacuacccuguccacccccucugcaaug-3', and c) for 5'-UTR element two 5'-agccctaagtgaccagctacagtcggaaaccatcagcaagcaggtcattgttccaac-3'.. In addition, a negative control biotinylated probe (scrambled sequence) was used as described to identify and eliminate non-specific RNA binding proteins. The biotinylated probes were immobilized to streptavidin agarose (Pierce Biotechnology, Rockford, IL) or streptavidin magnetic beads (Dynal, Lake Success, NY) overnight in a 1M NaCl-containing buffer as described (Ross et al., 1997). Beads were washed in high salt buffer to remove unbound probe, and then equilibrated in binding buffer. Concentrated cell extracts (50-250 mg protein) were prepared as described (Ross et al., 1997) from untreated or glucose-treated INS-1 or NIH3T3 cells. Extracts were incubated with immobilized biotinylated probes (1-5 mg of coupled probe) for 4-12 hours at 4 °C, washed, and proteins eluted in SDS-PAGE sample buffer. After separation by SDS-PAGE bands corresponding to proteins specifically bound to probes are excised and subjected to MALDI and protein sequencing.

To specifically confirm binding of polypyrimidine tract binding protein (PTB) to the preproinsulin 3' UTR, eluted PTB was analyzed by Western blot using commercially available PTB antibody (Figure 7). Both recombinant PTB and native PTB derived from INS-1 cell lysates was evaluated for binding.

Figure 8 illustrates the current paradigm of glucose-regulated RNA binding protein binding of RBP1 (also known as PTB) to the 3' UTR of the preproinsulin mRNA, as well as putative binding of other unidentified RBP1 RNA binding proteins. The 5'-UTR of preproinsulin mRNA contains a secondary (stem-loop) structure (ΔG= -10.8 kcal/mol) that is similar to structures found in other mRNAs that undergo regulation of biosynthesis at the translational level. Furthermore, the stem-loop structure is conserved in mammalian preproinsulin mRNAs. The 5'-UTR alone can function as the glucose-response element. When both 5'- and 3'-UTRs are present, there is an even greater response to glucose. In addition, the glucose-stimulated translation is pancreatic beta cell-specific, since no glucose response is observed in non-beta cells. This strongly suggests the involvement of glucose-regulated RNA

binding proteins working via the 5'-UTR. In its simplest form the available data suggest a model in which at low or resting glucose levels, an RNA binding protein(s) is bound to the 5'-UTR of the preproinsulin mRNA and represses its translation. Increased glucose concentrations cause a change in the abundance or in the affinity of the RNA binding protein(s) for the preproinsulin 5'-UTR, thus relieving the repression and allowing enhanced translation of preproinsulin mRNA (Figure 8).

Method 2: Direct Affinity-Tagging Of mRNA With An RNA-Epitope

An direct affinity-tagging of mRNA with an RNA-epitope assay is illustrated in Figure 6. This method is based on antibody-recognition of a unique RNA stem loop structure. The well-characterized antibody α-g10 (a.k.a. α-T7-tag) is raised against the N-terminus of g10 fusion protein by standard methods. This antibody is used to screen a complex library of degenerate RNAs (10^6 molecules) representing various stem loop structures. Following stringent washing conditions, a single 40 nucleotide RNA species is identified (D10) that was specifically recognized by α-g10. Upon further characterization, the D10 RNA is shown to mimic the peptide antigen, thus one can use the peptide for competition or elution. When the RNA-epitope is inserted into an mRNA, the RNA epitope-tagged mRNA could be specifically recovered from a mixture of total cellular mRNAs using α-g10. Furthermore, the antibody alone has no reactivity with total eukaryotic cellular mRNAs.

The D10 RNA-epitope tag is placed at the end of the 3'-UTR of the gene for Nkx6.1 and preproinsulin. This is accomplished by PCR cloning the tag into the full-length cDNAs for Nkx6.1 or preproinsulin (obtained by PCR cloning). These constructs are used for 1) generating *in vitro* transcripts for competition and affinity reagents, and 2) overexpression of Nkx6.1 or preproinsulin in a mammalian cell culture model followed by recovery of the RNA epitope-tagged mRNA from cell extracts with α-g10.

For the preproinsulin studies, the D10 RNA epitope-tagged preproinsulin cDNA is subcloned into pcDNA3.1neo and used to transfect MIN-6, α-TC1.6 and NIH3T3 cells. Transiently transfected cells as well as establish stable transfectants (selected with Neo) are

examined. Expression of the tagged mRNAs is under the control of the CMV promoter. Once expression of the tagged mRNA is confirmed by RT-PCR, extracts were prepared as described above from cells incubated in low or high glucose. Mock transfected cells are also examined.

Construction and transfection into the various cell-types of a D10 RNA epitope-tagged Nkx6.1 is performed in a similar manner. For analysis, the RNA epitope-tagged mRNAs are isolated from the extracts using immobilized α-g10. Proteins in these complexes are eluted with SDS-PAGE sample buffer or using antigenic peptide (NH_2-MASMTGGQQMGRC-COOH), which is previously shown to compete for the D10 epitope. A comparison of protein profiles obtained from the various cell extracts (including mock transfected cells) identified unique protein bands. The eluted proteins are processed as described in Example 1 above to obtain peptide sequence. One variation on this procedure included D10-tagging of a fragment of the full-length mRNA (e.g., the 5'- or 3'-UTR alone containing the D10 epitope).

A comparison of RNA binding protein expression profiles from α-TC1.6 cells, pancreatic beta cells (which express both homeodomain transcription factor Nkx6.1 mRNA and protein), and NIH3T3 cells is performed to identify cell-type specific RNA binding proteins using RiboMap™. These RNA binding proteins represent candidate proteins that control Nkx6.1 expression.

RAS™ is then performed using antibodies to these candidate RNA binding proteins and the resulting functional clusters analyzed for Nkx6.1 mRNA expression. A functional cluster containing Nkx6.1 mRNA contains other mRNAs that are coordinately regulated, and perhaps code for proteins involved in development of the endocrine pancreas and/or pancreatic beta cell differentiation. Proteins that bind to the 5'-UTR of Nkx6.1 mRNA can also be purified.

Specificity and mapping of RNA binding protein binding elements

In order to verify potential RNA binding proteins and their binding specificity, competition experiments using immobilized binding sites (either biotinylated probes or D10 epitope-tagged probes generated by *in vitro* transcription) are performed. For example, the specific binding site is immobilized with either streptavidin agarose or α-g10 agarose and incubated with cell extracts or a recombinant RNA binding protein according to art known methods. The binding reactions are carried out in the absence or presence of increasing concentrations of control or competing non-biotinylated or non-tagged probes (synthetic oligonucleotides or generated by *in vitro* transcription, as described above). Binding is analyzed by 1) electrophoretic mobility shift assays as described in the art and/or 2) SDS-PAGE followed by Coomassie staining, to detect the presence or absence of RNA binding protein bands. RAS™ may also be performed as a third verification procedure. In this case antibodies raised against the RNA binding protein are used to immunoprecipitate complexes as described above and microarray analysis is performed to identify the associated mRNAs, one of which should be the original endogenous target mRNA.

Example 3: Analysis of RNA Binding Protein Expression and Associated mRNAs in Human Adipocytes and Preadipocytes

Adipocytes have long been considered a primary location for glucose disposal and energy storage in the form of triglycerides (fat). There is growing evidence that adipocytes comprise a critical endocrine tissue that not only responds to insulin through glucose uptake and lipogenesis, but also synthesizes and secretes a variety of signaling molecules involved in systemic energy homeostasis. An analysis of RNA binding proteins and their associated mRNAs and mRNP complex-associated proteins and their role in gene expression in adipocytes provides a better understanding of adipocyte function and can provide targets for therapeutics that treat conditions associated with aberrant glucose metabolism. A flow chart for an exemplary adipocyte analysis is provided in Figure 9.

RNA binding proteins that are enriched in mature adipocytes vs. preadipocytes in lean individuals (BMI < 24) were identified as follows. Briefly, human preadipocytes were harvested from elective liposuction from three lean individuals. A portion of the preadipocytes were

differentiated in culture to mature adipocytes (Zen Bio, RTP, NC). The expression pattern of RNA binding proteins in mature adipocytes was compared to the expression pattern of RNA binding proteins in preadipocytes using a microarray chip, Ribochip™ V.1 array (MWG Biotech, Germany) according to the method described in Example 1. The Ribochip™ contains approximately 1400 distinct RNA binding proteins.

Figure 10 is a graphical representation of differentially expressed RNA binding proteins in preadipocytes compared to mature adipocytes. Eleven RNA binding proteins were identified that were enriched 2-fold or more in the mature adipocytes. Figure 11A is a graphical representation of certain RNA binding proteins that are upregulated in mature adipocytes as compared to preadipocytes. Figure 11B is a graphical representation of certain RNA binding proteins that are downregulated in mature adipocytes as compared to preadipocytes.

The RNA binding proteins identified as being up- or down- regulated two-fold or more were identified by the methods described in Example 1. Figure 12 provides a list of those RNA binding proteins. These data provide a refined list of candidate RNA binding proteins for further validation for participation in an adipocyte function pathway (e.g., maturation or differentiation) and/or glucose metabolism pathway and for the isolation of associated mRNP complex-associated proteins and associated mRNAs.

The effects of insulin or the pancreatic beta 3 agonist BRL-37344 on RNA binding protein expression in human mature adipocytes was also examined. Mature adipocytes from lean individuals were obtained as described above and treated with 100 nm insulin or 1μM BRL-37344. Cell lysates were prepared and RNA binding proteins were identified using Ribochip™ analysis as described above.

The RNA binding protein expression of insulin-treated adipocytes was compared to the RNA binding protein expression of untreated adipocytes. Figure 13A is a graphical representation showing the up- and down-regulation of the RNA binding proteins that are expressed in mature adipocytes in response to insulin or the pancreatic beta 3 inhibitor. Figure 13B shows another graphical representation of RNA binding proteins that are up- or down-

regulated 1.5-fold or more after treatment of mature adipocytes from lean individuals with insulin. Approximately 50 RNA binding protein genes were responsive to insulin (i.e., they were either up or down regulated at least 1.5-fold). RNA binding proteins were identified using Ribochip™ analysis as described above. Figure 14 provides a list of RNA binding proteins that are increased 2-fold or more in mature adipocytes from lean individuals treated with insulin. These data provide a refined list of candidate RNA binding proteins for further validation for participation in an adipocyte function pathway (e.g., maturation or differentiation) and/or a glucose metabolism pathway and for the isolation of associated mRNP complex-associated proteins and associated mRNAs.

In addition, the expression pattern of RNA binding proteins in mature adipocytes from three lean individuals was compared to the expression pattern of RNA binding proteins in mature adipocytes from three obese individuals (BMI < 30). Preadipocytes were obtained by elective liposuction and cultured as described above. Adipocytes from obese individuals showed an altered pattern of RNA binding protein expression. For example, only 4 of the 11 RNA binding proteins enriched in the adipocytes from lean individuals were present in the adipocytes from the obese individuals. Thus, 7 out of the 11 RNA binding protein genes are aberrantly regulated in obese individuals and are not induced by insulin.

In another experiment, the RNA binding protein expression in preadipocytes from obese individuals was compared to expression in mature adipocytes in obese individuals. Preadipocytes and adipocytes were obtained from obese individuals as described above. RNA binding proteins were identified using Ribochip™ analysis as described in Example 1. Figure 15 illustrates a list of 14 RNA binding proteins that were induced 2 fold or more in mature adipocytes from obese individuals as compared to preadipocytes from obese individuals. These data provide a refined list of candidate RNA binding proteins for further validation for participation in an adipocyte function pathway (i.e., maturation or differentiation) and/or glucose metabolism pathway and for the isolation of associated mRNP complex-associated proteins and associated mRNAs.

Example 4: Analysis of RNA Binding Protein Expression in Rat Pancreatic Beta Cells Treated with Insulin

The effect of glucose on RNA binding protein expression in rat pancreatic beta cells was examined. A derivative of the INS-1 rat pancreatic beta cell line, clone 832/13, was chosen because of its ability to mimic many of the normal functions of pancreatic islets. Whereas INS-1 cells respond to glucose treatment with a 2-4 fold increase in insulin secretion, clone 832/13 is induced 8-13 fold by glucose treatment. The 832/13 cell line was treated with glucose and RNA binding proteins that interact with the 5' and 3' UTRs of the proinsulin mRNA were isolated. In particular, RNA binding proteins that bind with high affinity and specificity to the UTR elements and whose binding or abundance is also glucose inducible are most likely to be the mediators of post-transcriptional regulation of proinsulin. Briefly, 832/13 cells were grown to near confluence, shifted to low glucose (3mM) for 1 hour 18 hours prior to assay (T = -18 hours), and treated for 2 hours (T = 0 to T= 2 hours) with fresh medium containing 3mM or 15 mM glucose. Insulin secretion was measured by ELISA (ICN Pharmaceuticals) for 24, 48, 72 hours and expressed as ng/mL. Fold-induction of insulin levels was determined as a measure of the difference between insulin secreted in response to low glucose concentration versus insulin secreted in response to high glucose concentration. Cells were harvested and cytoplasmic extracts were prepared as described in Example 1 for immediate use or storage at -80° C. Lysates derived from untreated primary rat and human islets were also evaluated as controls.

Preferably, the binding activity should be readily distinguishable as one, or at most, a few prominent RNA binding proteins, and not accompanied by a large number of mRNP complex-associated proteins. In addition, the amount of RNA binding protein is a key parameter for ensuring unambiguous sequence assignment for the bound RNA binding protein. Binding and washing conditions can be altered according to art known methods to optimize high affinity and sequence-specific interactions. For example, a stepwise increase in the concentration of monovalent or divalent cations (e.g., Na^+, K^+, Li^+, Ca^{2+}, Mg^{2+}) can be used to select for high affinity interactions. Additionally, unrelated nucleic acid sequences can be used to either pre-clear cytoplasmic lysates or as a control for non-specific RNA binding. Secondly, in this particular system, RNA binding activity is thought to be glucose-inducible, which provides an

important selection tool for the assignment of regulatory RNA binding protein. Thus, the UTR binding activities should fluctuate with glucose induction.

Alternatively, a yeast-based 3-hybrid screening (Bernstein et al. (2002) Methods 26:123-41) may be used to isolate RNA binding proteins. Briefly, activation of a reporter gene in yeast is dependent upon the interaction between a test RNA (e.g., the proinsulin UTR) and candidates from a library of co-expressed RNA binding protein fusion proteins. From a commercial standpoint, the development of either or both techniques can be used to define specific regulatory RNA binding proteins associated with known or unknown mRNAs or mRNA fragments.

Example 5: Identification Of mRNA Subsets Associated With PTB Complexes That Are Co-Regulated With Insulin mRNA Expression In Rat Glucose-Responsive Pancreatic Beta Cells (INS-1) Using cDNA Arrays (RAS™)

An *in vitro* cDNA array RAS™ assay was used to identify the subset of mRNAs associated with PTB and other RNA binding protein complexes, for example, in rat glucose-responsive islets (INS-1) cells.

Cloning and Expression of RNA Binding Protein Genes that Regulate Insulin

Regulatory RNA binding proteins that bind to the 3' UTR of the proinsulin gene were identified according to Example 2. RNA binding protein cDNAs were cloned and expressed in bacterial vectors as tagged or fusion proteins (e.g., 6X His or glutathione-S-transferase (GST)). For example, the RNA binding protein named polypyrimidine tract binding (PTB) protein binds to the 3' UTR of proinsulin mRNA. The human PTB cDNA was cloned into a pGEX4T vector, which contains a GST affinity tag, and expressed in *E. coli* cells in the presence and absence of glucose. The GST-PTB fusion protein was purified from bacterial lysates using the GST affinity tag, as described above

Isolation of Co-Regulated mRNAs

The GST-PTB fusion protein was used to screen for mRNAs that bind to PTB. Briefly, the GST-PTB fusion protein was bound to a glutathione sepharose support through the GST

linkage according to standard methods. INS-1 cells were cultured as described in Example 2. Cytoplasmic lysates containing mRNA from glucose-treated INS-1 cells or from primary islets were exposed to the bead-bound GST-PTB fusion protein. Messenger RNAs containing UTR elements that bind to PTB were retained on the beads. Ionic conditions for binding and washing were altered to select for high affinity binding of mRNAs to PTB or other RNA binding proteins, as described above.

Bound mRNAs were selectively eluted with glutathione, which competes with GST to displace the mRNA-RNA binding protein complexes from the beads. Glutathione elution enables the selective elution of only those mRNAs that are bound to the RNA binding protein, and minimizes contamination with mRNAs that are non-specifically associated with the sepharose matrix. As a positive control, eluted mRNAs were enriched for the presence of proinsulin mRNA, which was directly assessed using quantitative PCR, according to standard methods.

After assessing the overall gene expression profile of the RBP1 mRNA complexes, they were immunoprecipitated and captured mRNAs were identified on cDNA arrays to quantitate the full spectrum of bound mRNAs. Briefly, cDNA array analysis was performed using Agilent Human Unigene 14K (Agilent, Palo Alto, CA) or MWG Pan Human 10K (MWG Biotech, Inc., High Point, NC), two-color fluorescent high density microarrays. Briefly, RNA was extracted from INL-1 cell lysates and used to produce reverse transcribed probes according to standard methods. A pooled set of primers, complementary to the genes represented on the array, was used for the reverse transcription probe synthesis, which was radiolabeled with $^{32}P\alpha$-dATP. The radiolabeled probe was purified by passage over CHROMA SPIN™-200 columns (Clontech, Inc., Palo Alto, CA) and incubated overnight with an array membrane using EXPRESSHYB™ hybridization solution (Clontech, Inc., Palo Alto, CA) according to the manufacturer's protocols. Following hybridization, the array membrane was washed and visualized on a phosphorimaging screen (Molecular Dynamics, Sunnyvale, CA).

Phosphorimages were scanned using the Molecular Dynamics STORM 860 System at 100 micron resolution and stored as files. Images were analyzed using ATLASIMAGE™ 1.0

and 1.01 software (Clontech, Inc., Palo Alto, CA). The signal for any given gene was calculated as the average of the signals at two duplicate cDNA spots. As described in the ATLASIMAGE™ 1.0 software manual (Clontech, Inc., Palo Alto, CA), a default external background setting was used in conjunction with a background-based signal threshold to determine gene signal significance. The signal for a gene was considered significantly above background if its adjusted intensity (total signal minus background) was more than two-fold the background signal. Comparisons of multiple cDNA array images were performed using an average of all the gene signals on the array (global normalization) to normalize the signal intensity between arrays. The initial alignment of these arrays was facilitated by spiking the hybridization reaction with radiolabeled lambda phage markers that hybridized with six DNA spots on the bottom of the array membrane. Once the alignment register was established, subsequent blots did not require the use of spiked lambda markers for orientation. cDNA array images and overlays were prepared using ADOBE PHOTOSHOP® 5.0.2 (San Jose, California, USA).

RBP1 complexes isolated from rat glucose-responsive islets (INS-1 cells) contained the mRNA classes listed in Table 2.

Table 2: Gene List for RBP1 Functional Cluster

14 Kinases*
11 Transporters
8 Phosphatases
18 Channels**
4 Peptidases/Proteases
33 Receptors
3 Transcription Factors
8 Transferases

*8/14 genes have no previous role in Glucose Stimulate Insulin Secretion

**14/18 genes have no previous role in Glucose Stimulate Insulin Secretion

Figures 16A and 16B provide lists of the mRNAs that are associated with the PTB RNA binding protein. It is notable that many of the ion channel proteins identified on the PTB cluster

were not previously identified as participating in glucose metabolism (Figure 17). These proteins represent targets for new therapeutics that may be used to regulate a pathway that participates in glucose metabolism or other pancreatic beta cell function. Figure 2 illustrates some of the known pathways that participate in insulin secretion in pancreatic beta cells, indicating some of the proteins encoded by mRNAs listed in Figures 16A and 16B.

A representative set of mRNAs enriched over five fold in the RNA binding protein-bound fraction was evaluated by gel retardation analysis to confirm that the bound mRNAs contain specific sequence elements that participate in RNA binding protein regulation.

Confirmation That Candidate RNA Binding Proteins That Are Co-Regulated With Proinsulin mRNA *In Vitro* Bind to the Same RNP Complex *In Vivo*

Sharing common RNA binding protein-binding elements with the proinsulin gene may represent a common requirement for the set of genes that are potentially post-transcriptionally co-regulated in concert with proinsulin. The identification *en masse* of binding sites for regulatory RNA binding proteins provides a provisional set of co-regulated genes that can be compared to those identified from endogenous RNP complexes. To confirm their association with the proinsulin regulatory RNA binding proteins *in vivo*, tagged versions of PTB and the other putative 5' and 3' UTR-binding RNA binding proteins are expressed as tagged fusion proteins in INS-1 cells. Using flag- or G10-epitope tags, the three putative RNA binding proteins (PTB and two unknown RNA binding proteins) are stably selected and expressed in INS-1 cells. The addition of the epitope tags streamlines the ability to immunoprecipitate the RNP complexes from the cells, since under most circumstances the epitope is not buried within the complex. Following stable selection of the tagged RNA binding proteins, RNP complexes containing the three putative proinsulin regulatory RNA binding proteins being analyzed by *in vitro* binding are immunoprecipitated.

For comparison to the mRNA pools generated by *in vitro* binding, RNA was isolated from the immunoprecipitated *in vivo* RNP complexes and analyzed by a combination of microarray and quantitative PCR.

The combined *in vitro* and *in vivo* analysis of RNP complexes offers a powerful approach to the study of post-transcriptional regulation. The comparative analysis identifies the set of genes being coordinately regulated in conjunction with the proinsulin gene in response to glucose. These data provide a roadmap of the regulatory, metabolic, and signalling pathways that act in concert to orchestrate the proper production of insulin, for example. Analysis of dynamic changes in RNP complex composition in islet cells will lead to the identification of novel diagnostic biomarkers and a collection of compelling therapeutic targets for modulating insulin production or other gene involved in glucose metabolism. However, to confirm that the mRNAs identified are in fact co-regulated with proinsulin mRNA, their associations need to be confirmed *in vivo*, for example, using knockout analyses as described in Example 7.

Example 6: Validation of Functional Role for Genes in the RBP1 Functional Cluster

To confirm that a gene identified in an mRNP plays a direct role in the etiology of a disease or other phenotype being studied, candidate target genes are chosen for inhibition studies, e.g., using antisense RNA or RNAi. For example, for each candidate therapeutic gene, one or more short DNA segments representing the coding sequence of that gene, is individually cloned into a plasmid vector in the sense or antisense direction, downstream of an appropriate promoter, such as a U6 polymerase III promoter or RNAse P RNA H1. Plasmid vectors may be constructed that contain two or more short DNA segments of one or more candidate therapeutic genes in the sense and antisense directions, downstream of a U6 polymerase III promoter or RNAse P RNA H1. Alternatively, one may construct an RNAi by annealing chemically synthesized complementary 22 bp RNAs (Dharmacon, Lafayette, CO).

Following transfection of the vector or double stranded RNA into cultured cells, phenotypic characteristics are evaluated to determine the effect of inhibiting the expression of the candidate target gene(s). In addition, to verify inhibition of gene expression at the RNA and protein levels, Northern blots, quantitative RT-PCR, Western blot, or other analytical assay of time course experiments are performed to demonstrate the efficacy and duration of inhibition for the individual genes.

Transfections can result in transient expression for one to five days. Alternatively, vectors expressing RNAi can be stably expressed in cultured cells by co-transfection and selection with a dominant selectable marker, such as neomycin. As alternatives to the use of RNAi, traditional antisense DNA or vectors expressing dominant negative forms of targets of interest can be used. Antisense and dominant negative genes can be delivered by direct DNA transfection or through the use of virus vectors including, but not limited to, retroviruses, adenoviruses, adeno associated viruses, baculoviruses, poxviruses, and polyomaviruses. The biological system of study chosen to demonstrate the role of a gene in disease or cellular phenotype is based upon knowledge in the art of the biological system, including a cell culture or animal model system, that mimics relevant biological features.

Knockout of RNA Binding Protein RBP1 Expression

Although inhibition of RNA binding proteins that bind and regulate multiple mRNAs is likely to have a more drastic effect on the phenotype of the cells, this procedure remains an important control to verify the critical importance of the mRNP complex. RNAi knock-out experiments were used to knock out the expression of the RNA binding protein RBP1. The results validated the utility of the invention for isolating functionally related groups of genes and rapidly identifying and validating novel pathway components relevant to drug discovery. RNAis and transfection materials were obtained from Darmachon. Smart pools of rRNAi were made by propietary algorithm to design up to 20 RNAis against the gene of interest to increase the likelihood of success. RNAis were transfected according to the manufacturer's protocols for transfection reagents. Results were verified by QRTPCR or Western blot. RNAi mediated knock-out of RBP1 expression lead to increased basal insulin secretion levels and decreased cellular response to glucose. Readout is insulin secretion at basal levels (no glucose stimulation) versus insulin secretion upon high glucose stimulation.

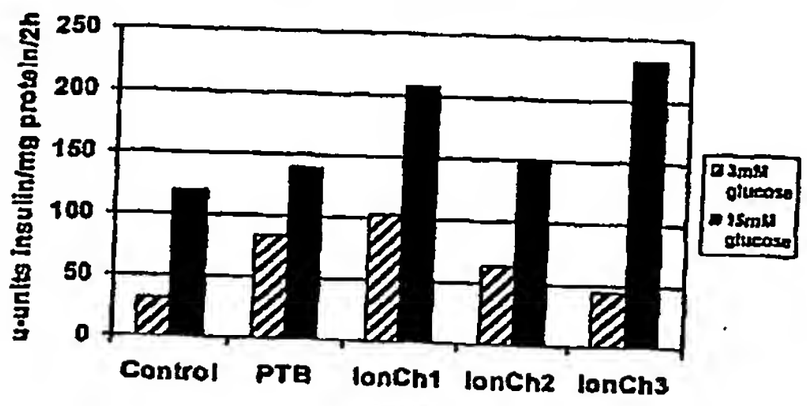
Knockout of the Expression of Two Ion Channel Protein Associated with RBP1

RNAi knock-out of two ion channel proteins resulted in significantly increased levels of basal insulin secretion. Knock out of two potassium channels caused extreme increase in basal insulin secretion levels, suggesting these channels play a functional role in the process.

These two potassium channel proteins were not previously implicated in regulating insulin secretion or pancreatic beta cell function. This is significant, since the action of a class of diabetes drugs (sulfonyureas or gliburides like GLUCOVANCE) act by inhibiting a K^+ channel on the pancreatic beta cell. This inhibition leads to membrane depolarization, which allows calcium to enter the cell and stimulate release of intracellular secretory granules filled with insulin. These drugs act by increasing overall and basal insulin secretion, thereby controlling high glucose levels (hyperglycemia). These results suggest that there are additional K^+ channels that may work in this process and provide candidate targets for new diabetes drugs.

RNAi and target validation. Initial target validation screens center around the RNA interference (RNAi) scheme. In this system INS-1 cells were transfected with siRNA smartpools (Dharmacon) that are chosen based on a proprietary algorithm. The smartpools are very effective; at least a 50% reduction on gene expression is detected by 24-48hours post-transfection. As a first pass screen, and test of the RNAi system in the INS-1 cells, three ion channels (ionch1, 2, and 3) that have previously not been associated with glucose-stimulated insulin secretion (GSIS) were analysed. In addition, RNAi knockdown of PTB was also analysed to determine the effect of its decreased expression on GSIS. Finally, a mixed-sequence siRNA with no homology to any gene in the current rat database was used as a

Fig 5. Effect of PTB and IonCh siRNAs on GSIS



control. Expression of target genes in untreated, control transfected and sequence-specific siRNA-transfected cells is assessed by QPCR and/or immunoblotting. Compared to cells transfected with the control siRNA, transfection of INS-1 cells with siRNA to PTB or ionch1 resulted in significantly elevated basal levels (3mm glucose) of insulin secretion (Fig 5). The ability of glucose to stimulate insulin secretion in sirna PTB cells was dimished, and although siRNA ionch1 cells had an overall reduced fold-stimulation by glucose, there was a significantly elevated level of total secreted insulin. Basal secretion in ionch2 or ionch3 transfected cells was only slightly elevated and showed normal or slightly elevated glucose stimulation. In addition extensive time course expreiments and glucose dose response and ability to respond to other secretagogues such as sulfonylureas, GLP-1 and fatty acids are performed.

Over-expression of Target Proteins

Alternatively, cells can be transfected with nucleic acids encoding target proteins or treated with a transcriptional enhancer for a gene encoding a target protein of interest, in order to overexpress a particular target protein identified by the methods of the invention. These systems would then be subject to biological assays (e.g., glucose-stimulated insulin secretion), as described above.

In vitro validation

As indicated above, it has been proposed that PTB binds to a pyrimidine-rich site in the 3' UTR of the proinsulin mRNA in a glucose dependent manner. To validate that PTB indeed is responsible, an oligonucleotide capture is used to demonstrate high affinity, sequence specific binding to the 3'UTR element. Briefly, biotinylated oligoribonucleotides encoding the entire 3' UTR (53nt) or 5' UTR (58nt) of rat preproinsulin, and controls are bound to streptavidin-agarose beads and exposed to cell or tissue lysates. Following washing of the beads (low to high salt) , selectively bound PTB is detected by elution with SDS PAGE sample buffer and immunoblot. Both endogenous PTB from INS-1 cells and recombinant GST-PTB (data not shown) can specifically associate with the 3'UTR and that the association is stable to 300 mM NaCl. Using this system, the sequence specificity and

the ability of glucose to regulate PTB binding to the preproinsulin mRNA is examined. In these experiments cell extracts are prepared from INS-1 cells that have been incubated under low (3mM) to high (15mM) glucose. Control and sequence-specific biotinylated oligos are incubated with these extracts, the beads washed and proteins bound to the beads analyzed by immunoblotting with a monoclonal antibody to PTB (Zymed). It is expected that if glucose can modulate the binding of PTB to the preproinsulin UTR an increase/decrease in PTB protein associated with the sequence-specific oligo is detected.

In vivo validation

To validate a functional role for PTB and the novel glucose-regulated RBP, a newly emergent RNA interference techniques (RNAi) is used to acutely reduce expression of the two genes in INS-1 cells. Pools of siRNAs (SmartPools from Dharmacon) designed specifically for PTB and the novel RBP gene are transfected into INS-1 cells at various concentration (1nM -200nM) using the RNAi transfection reagent TransIT-TKO (Mirus). Control siRNAs that do not have sequence homology to any current gene in the rat database are included in all experiments. Following transfection, cells are harvested at various time points 24-96 hours post transfection to verify reduction in expression of the RNA binding proteins at both the RNA and protein levels (antibody permitting). Following optimization of conditions for RNA binding protein suppression, cells are challenged with low and high glucose to determine affects on glucose stimulated insulin secretion (Elisa, Linco, Inc). It is expected that downregulation of PTB which binds to the preproinsulin mRNA should quantitatively or temporally affect insulin biosynthesis and/or secretion. This effect on insulin secretion is verified, which suggests that PTB and/or mRNAs associated with the cluster play a functional role in GSIS. According to the current model, in addition to PTB, at least one additional RBP involved in regulating insulin secretion or proinsulin biosynthesis is identified.

Example 7: Identification of Common Untranslated Elements in mRNAs identified Using RAS™

Following application of the RAS™ assay, the subpopulation of mRNAs that are present in mRNP complexes can be identified and examined for the presence of common UTR sequence

elements. Computational analysis for homology is not a reliable method for identifying Untranslated Sequence Elements for Regulation Codes (USER codes) because they are often dependent on secondary structure rather than just primary (sequence) sequence. In addition, the subpopulation of mRNAs can be examined for functional relationships. For example, each mRNA can be categorized by gene annotation and by known functions in functional genomics databases (*e.g.*, Locus Link (NCBI, Bethesda, MD), GO Database (Gene Ontology™ Consortium), Proteome BioKnowledge® Library (Incyte Genomics, Inc., Palo Alto, CA)). For example, if the protein used in the RiboTrap™ assay is involved in immune regulation, the other mRNAs found in the same mRNP complex can be analyzed for their role in immune regulation. However, the mRNA could be bound indirectly through a different RNA binding protein in the mRNP complex (*e.g.*, is assessed to the presence of USER code element in its UTR that recognizes the RNA binding protein or other known binding sites for RNA binding proteins.

A goal of the RAS™ assay is to identify mRNA populations in which the mRNAs have related structural features in their UTRs or the proteins encoded by the mRNAs have functional relationships. Among the related functions that are expected are a) involvement of encoded proteins in a common metabolic pathway, b) encoded proteins that are temporally co-regulated, c) encoded proteins that are similarly localized in or on the cell, d) encoded proteins that play a role in forming or regulating a biological machine (*e.g.*, a ribosome). The identification of complex traits and phenotypes that result from the expression of a set of functionally-related proteins would include such processes as cognition, cell-specific activation, inflammation, or differentiation. While proteins known to be involved in these complex processes are known from other studies, the majority of the functions remain largely unknown. One of the values of the invention is for discovering a larger set of proteins involved in these processes that could serve as alternative drug targets or surrogate markers.

Equivalents

The invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The foregoing embodiments are therefore to be considered in all respects illustrative rather than limiting on the invention described herein. Scope of the

invention is thus indicated by the appended claims rather than by the foregoing description, and all changes that come within the meaning and range of equivalency of the claims are intended to be embraced therein.

Incorporation by Reference

All publications and patent documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if the contents of each individual publication or patent document was incorporated herein.

We claim:

CLAIMS

1. A method for identifying a therapeutic target, the method comprising the steps of comparing expression of at least one protein in a first sample to expression of said protein in a second sample, wherein said protein is selected from the group consisting of an RNA binding protein or an mRNP complex-associated protein, and identifying a differentially expressed RNA binding protein or mRNP complex-associated protein as a therapeutic target.

2. The method of claim 1, further comprising the step of identifying a member selected from the group consisting of an mRNA encoding said protein, a gene encoding said protein, an mRNP complex comprising said protein, an mRNA associated with said mRNP complex, an mRNA associated with said protein, a gene encoding said mRNA associated with said mRNP complex, and a gene encoding said mRNA associated with said RNA binding protein, as a second therapeutic target.

3. The method of claim 1 or 2, wherein said first sample is obtained from an individual exhibiting a phenotype indicative of disease and wherein said second sample is obtained from an individual not exhibiting said phenotype.

4. The method of claim 3, wherein said phenotype is obesity.

5. The method of claim 3, wherein said phenotype is diabetes.

6. The method of claim 3, wherein said phenotype is hypoglycemia.

7. The method of claim 1 or 2, wherein said first sample comprises differentiated cells and said second sample comprises copies of undifferentiated cells.

8. The method of claim 1 or 2, wherein said sample scomprises an adipocyte or a pancreatic beta cell.

9. The method of claim 1 or 2, wherein said samples comprises a mature adipocyte.

10. The method of claim 1 or 2, wherein said samples comprise a preadipocyte.

11. The method of claim 1 or 2, wherein said samples comprise a hepatocyte.

12. The method of claim 1 or 2, wherein said RNA binding protein is a polypyrimidine tract binding (PTB) protein.

13. The method of claim 1 or 2, wherein said RNA binding protein is a selected from the group consisting of ribonuclease P (30kD), nuclear receptor coactivator 5, DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 28, RNA binding motif protein (Y chromosome, A1), LOC136197, exosome component Rrp46, zinc finger protein 85 (HPF4, HTF1), LOC134477, pterin-4 alpha-carbinolamine dehydratase, nuclear receptor co-repressor 2, IGF-II mRNA-binding protein 1,putative 28kDa protein, gonadotropin-regulated testicular RNA helicase, LOC56902 (hypothetical protein XP_095071), primase (polypeptide 2A (58kD)), bol (boule-like, Drosophila), LOC151613, small nuclear ribonucleoprotein polypeptide G, nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100), heterogeneous nuclear ribonucleoprotein D, LOC163412 (hypothetical protein XP_088868), ectonucleotide prophosphatase/phosphodiesterase 2, ribosomal protein S15a, LOC126205, huntingtin-interacting protein HYPA/FBP11, LOC138280 (similar to KIAA1138 protein), LOC165271 (similar to hypothetical protein DKFZp434B1727 from H. sapiens), ribonuclease (RNase A family, 2), human epididymis-specific 3 beta, LOC129715 (similar to tudor protein), ribosomal protein S21, 2',5'-oligoadenylate synthetase 3, ring finger protein 17 isoform short, LOC127164 (similar to TAR DNA binding protein), and 2',5'-oligoadenylate synthetase 1 (isoform E16).

14. The method of claim 1 or 2, wherein said first sample is treated with an agent selected from the group consisting of insulin, insulin-like growth factor-1 (IGF-1), β-adrenergic agonist, glucose, glucagon-like peptide-1 (GLP-1), fatty acid and thiazolidinediones and wherein said second sample is not treated with said agent.

15. A method for identifying a gene or gene product involved in a biochemical pathway in a cell, the method comprising the steps of:

a. identifying at least one protein in a cell, wherein said protein is selected from the group consisting of an RNA binding protein and an mRNP complex-associated protein that is differentially expressed in a first sample and a second sample, and wherein said differential expression is indicative that said protein is a therapeutic target;

b. isolating an mRNP protein complex comprising said protein and at least one mRNA; and

c. identifying a component of said complex as being involved in a biochemical pathway.

16. The method of claim 1 or 2, wherein said mRNA is selected from the group consisting of the genes listed in Figure 16.

17. The method of claim 15, further comprising the step of contacting said first sample with an agent that interacts with or regulates a component of said biochemical pathway.

18. The method of claim 15, wherein said agent inhibits said biochemical pathway.

19. The method of claim 15, wherein said agent enhances said biochemical pathway.

20. The method of claim 15, wherein said biochemical pathway is an insulin production pathway.

21. The method of claim 15, wherein said biochemical pathway is a lipogenesis pathway.

22. The method of claim 15, wherein said protein is a polypyrimidine tract binding (PTB) protein.

23. The method of claim 15, wherein said sample comprises preadipocytes, adipocytes or pancreatic beta cells.

24. A method for identifying an agent that interacts with or regulates a component of a biochemical pathway, the method comprising the steps of:

a. contacting a sample comprising a biochemical pathway with an agent;

b. preparing a ribonomic profile of said agent-treated sample, wherein said ribonomic profile comprises expression of at least one component of said biochemical pathway associated with at least one mRNP complex, said component selected from the group consisting of an RNA binding protein, an mRNA, and an mRNP complex-associated protein; and

c. comparing the level of expression of said component in said agent-treated sample to the level of expression of said component in a control sample, wherein a difference in said expression of said component is indicative that said agent is capable of interacting with or regulating said component.

25. A method for assessing the efficacy of an agent as a therapeutic in treating an individual having a disease associated with altered glucose metabolism, the method comprising the steps of:

a. contacting a sample from an individual having a disease with an agent;

b. preparing a ribonomic profile of said agent-treated sample, wherein said ribonomic profile comprises expression of at least one gene product associated with at least one mRNP complex, said gene product selected from the group consisting of an RNA binding protein, an mRNA, and an mRNP complex-associated protein; and

c. comparing the level of expression of said gene product in said agent-treated sample to the level of expression of said gene product in a control sample, wherein a difference in expression of said gene product is indicative that said agent is a therapeutic capable of treating said disease.

26. The method of claim 25, wherein said disease is diabetes.

27. The method of claim 25, wherein said disease is obesity.

28. The method of claim 25, wherein said disease is hypoglycemia.

29. The method of claim 25, wherein said sample comprises an adipocyte.

30. The method of claim 25, wherein said sample comprises a pancreatic beta cell.

31. The method of claim 25, wherein said cell is a hepatocyte.

32. A method for identifying a protein that regulates glucose metabolism, the method comprising the steps of:

a. measuring said expression of at least one gene product of a pancreatic beta cell sample, wherein said gene product is selected from the group consisting of an RNA binding protein, an mRNA associated with said RNA binding protein, or an mRNP complex-associated protein;

b. treating said pancreatic beta-cell sample with glucose, glucagon-like peptide-1 (GLP-1), fatty acid or a thiazolidinedione; and

c. measuring expression of said protein after treatment, wherein a difference in expression of said protein after treatment compared to expression of said protein before treatment is indicative that said protein regulates glucose metabolism.

33. A method of identifying gene products co-regulated with preproinsulin mRNA, the method comprising the steps of:

a. isolating an mRNP complex comprising a protein identified by the method of claim 1 or 2, and at least one mRNA or at least one mRNP complex-associated protein; and,

b. identifying said mRNA or said mRNP complex-associated protein, wherein said mRNA or said mRNP complex-associated protein are co-regulated with preproinsulin mRNA.

34. A method for identifying a protein that regulates insulin production, the method comprising the steps of:

a. contacting a pancreatic beta cell sample with at least one probe, wherein said probe is capable of binding to at least one protein, wherein said protein is capable of binding to the 3' UTR or 5' UTR of a preproinsulin mRNA;

b. separating said probe from said protein; and

c. identifying said protein.

35. The method of claim 34, wherein protein binds to a probe comprising a sequence selected from the group consisting of 5'-gaauaaaaccuuugaaagagcacuac-3', and 5'-cccaccacuacccuguccaccccucugcaaug-3', and 5'-agccctaagtgaccagctacagtcggaaaccatcagcaagcaggtcattgttccaac-3'.

36. An isolated composition of matter identified by the method of claim 24.

37. An isolated mRNA protein complex identified by the method of claim 15.

38. An mRNP complex-associated with diabetes, wherein said mRNP complex comprises a polypyrimidine tract binding (PTB) protein, and at least one mRNA associated with said polypyrimidine tract binding protein.

38. The method of claim 15, wherein said biochemical pathway is a glucose-uptake pathway.

ABSTRACT

The identification and evaluation of mRNA and protein targets associated with RNA binding proteins or mRNP complexes associated with glucose metabolism is described. In particular, the invention provides methods for identifying RNA binding proteins associated with glucose metabolism biochemical pathways and mRNAs that exhibit coordinated gene regulation across those pathways. Candidate targets are provided that are useful for treating diseases related to aberrant glucose metabolism, such as, for example, obesity and diabetes.

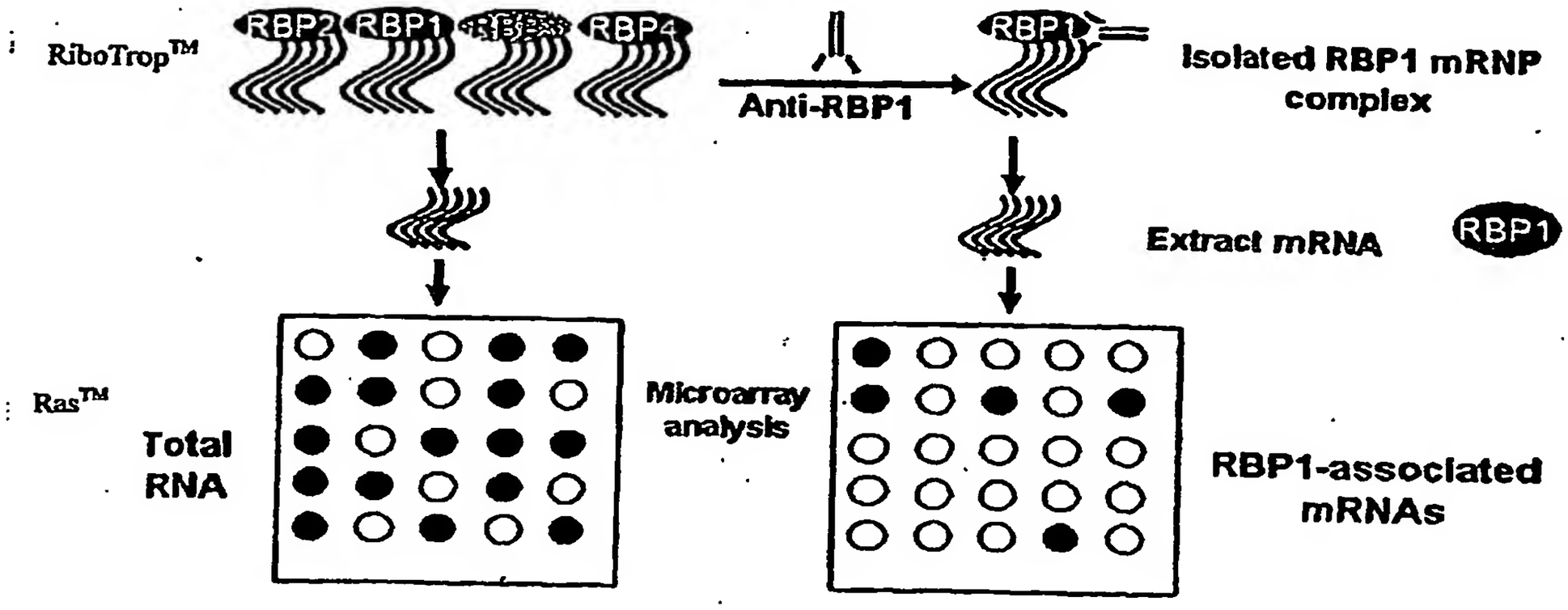

RiboTrop™
RBP2
RBP1
RBP3
RBP4
Anti-RBP1
RBP1
Isolated RBP1 mRNP complex
Extract mRNA
RBP1
Ras™
Total RNA
Microarray analysis
RBP1-associated mRNAs
•Global view of transcriptome
•Low sensitivity
•Complex Data Sets
•Enriched subset of mRNAs
•Higher Sensitivity
•Focus--Simplifies Data sets
•Many in same pathway, i.e. 'functionally related'
•Identify Novel Components

FIG. 1A

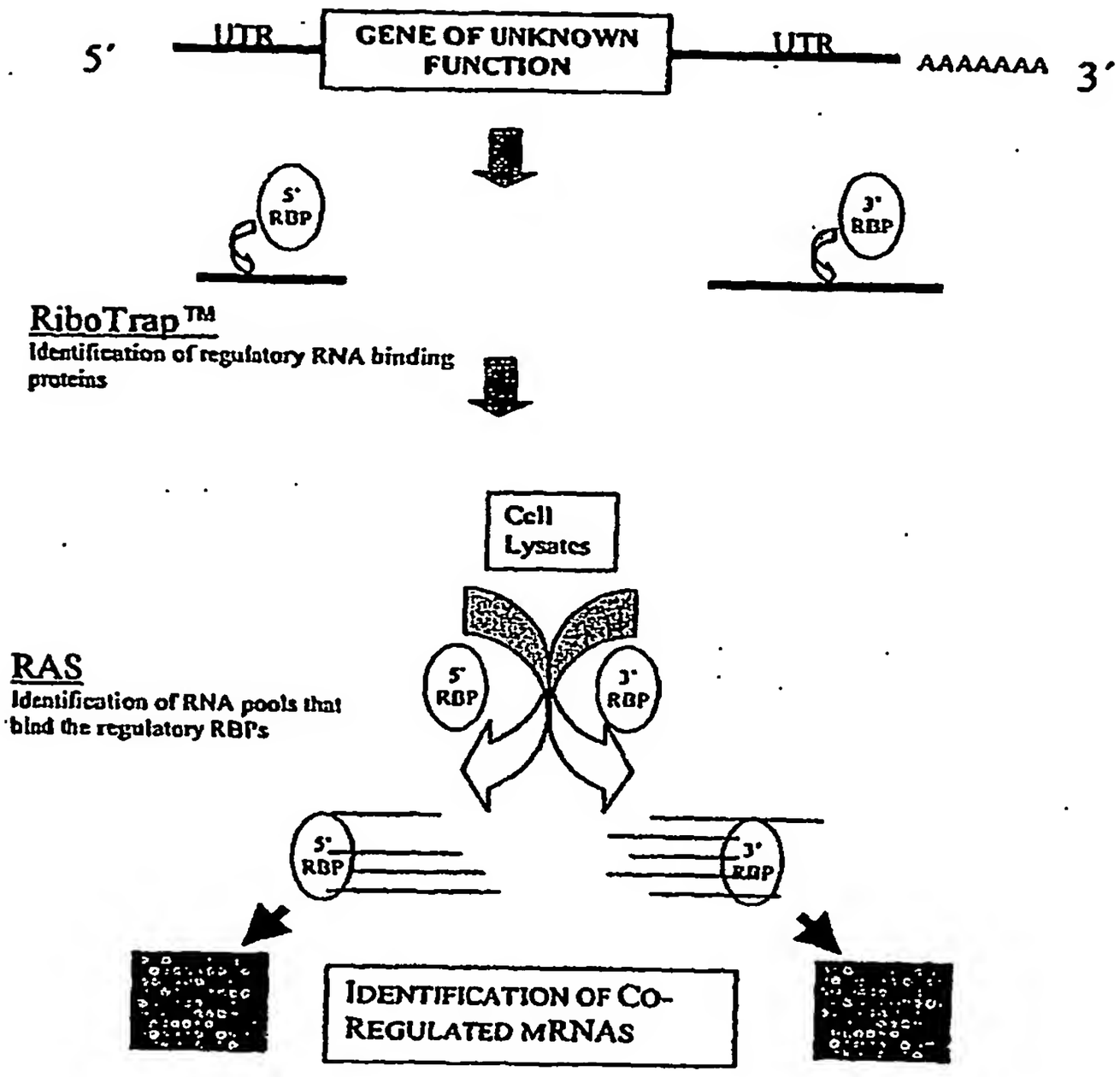

5'
UTR
GENE OF UNKNOWN FUNCTION
UTR
AAAAAAA
3'
5' RBP
3' RBP
RiboTrap™
Identification of regulatory RNA binding proteins
Cell Lysates
RAS
Identification of RNA pools that bind the regulatory RBPs
5' RBP
3' RBP
5' RBP
3' RBP
IDENTIFICATION OF CO-REGULATED mRNAS

FIG. 1B

Stimulus-Coupling Mechanism of Insulin Secretion in ß-Cells

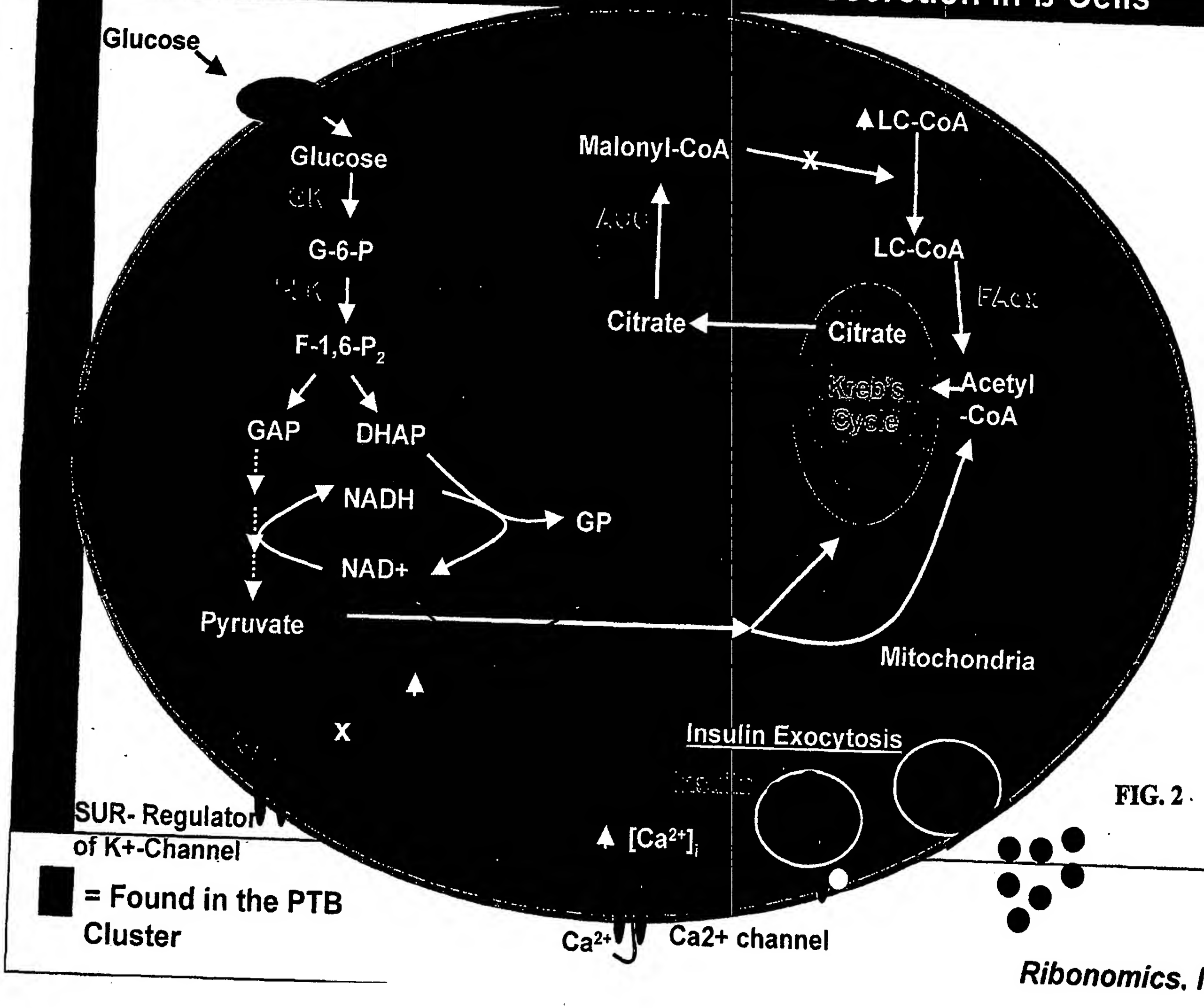


FIG. 2

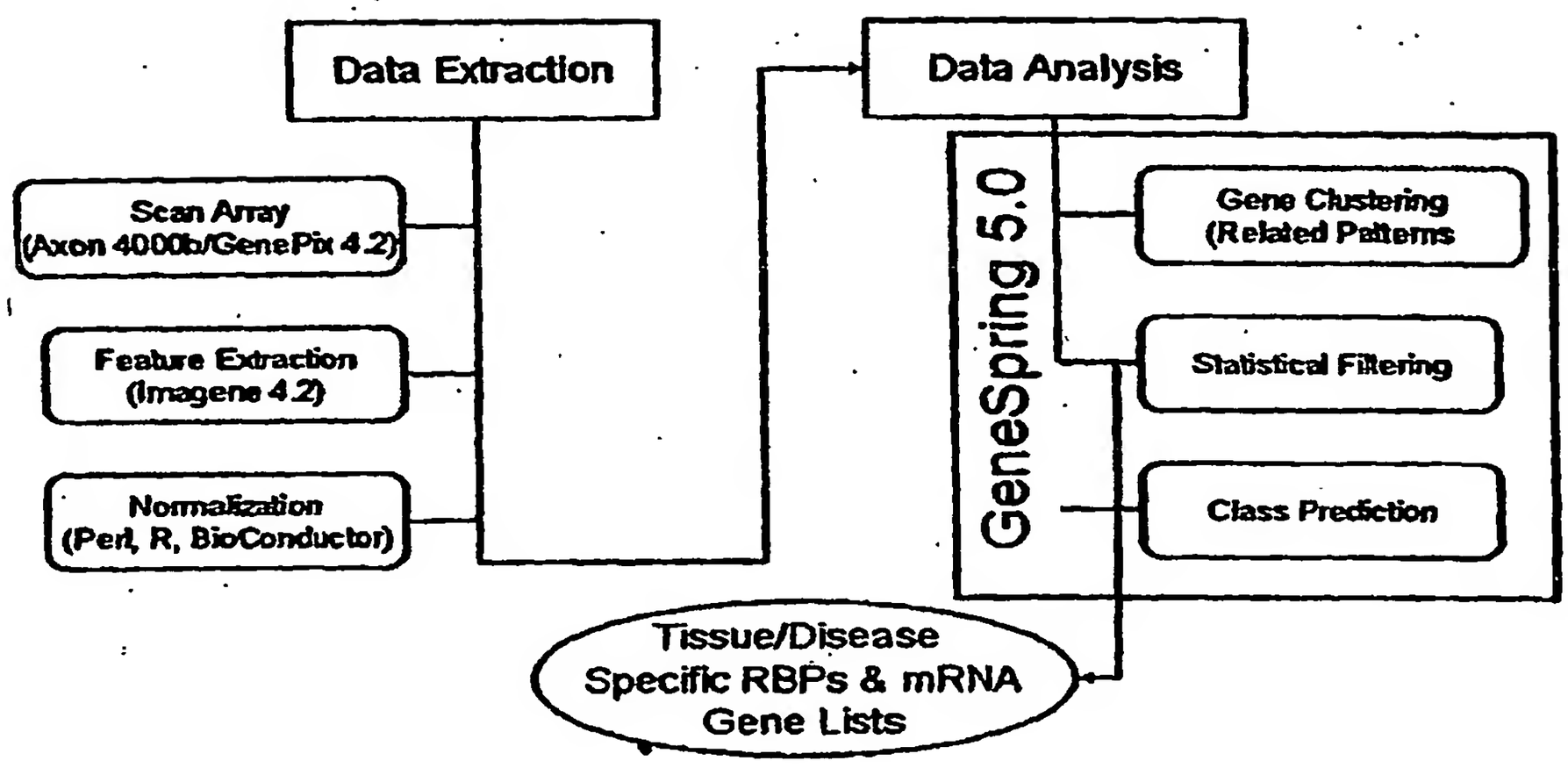

Data Extraction
Scan Array
(Axon 4000b/GenePix 4.2)
Feature Extraction
(Imagene 4.2)
Normalization
(Perl, R, BioConductor)
Data Analysis
GeneSpring 5.0
Gene Clustering
(Related Patterns
Statistical Filtering
Class Prediction
Tissue/Disease
Specific RBPs & mRNA
Gene Lists

FIG. 3

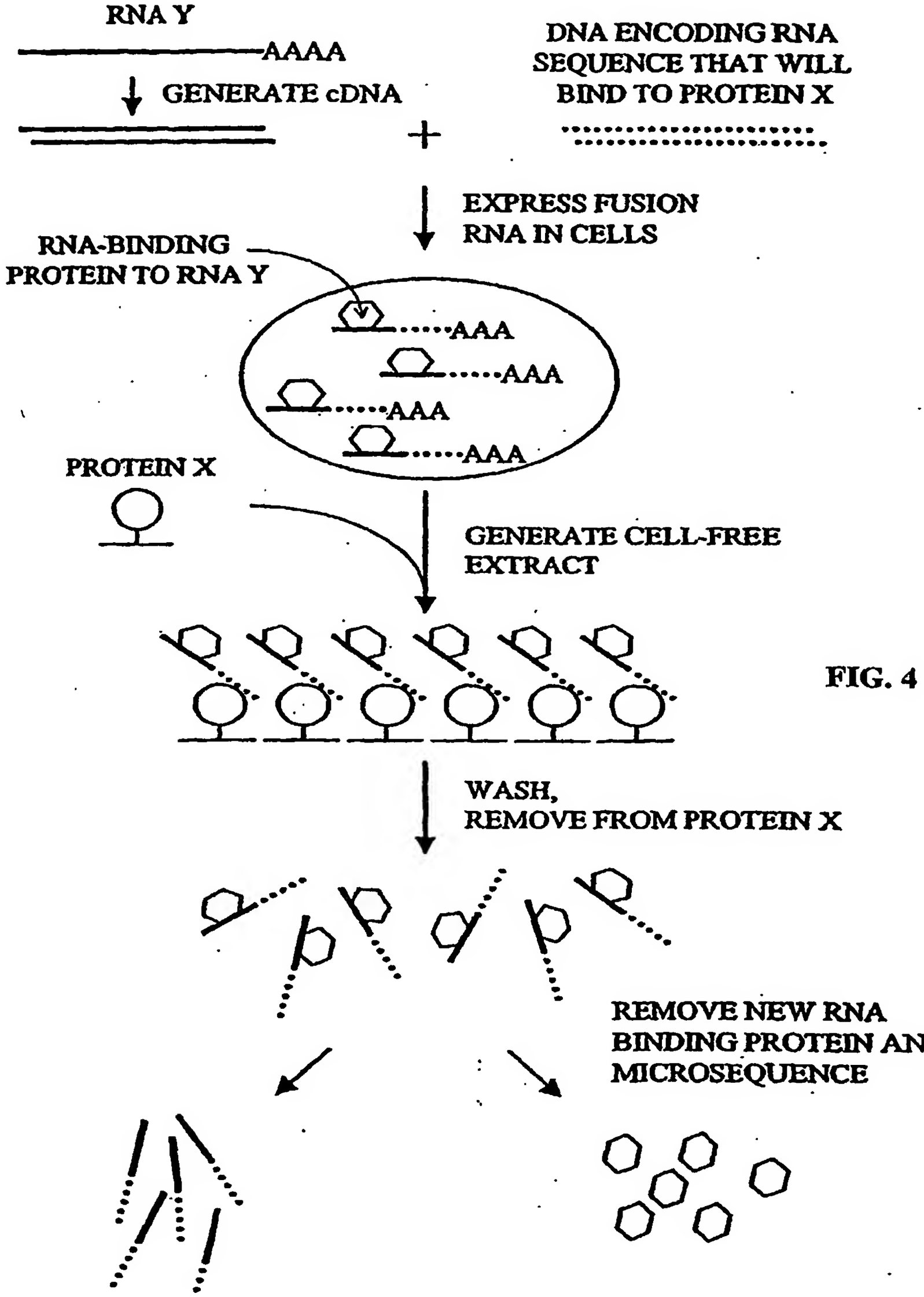

RNA Y
AAAA
GENERATE cDNA
DNA ENCODING RNA SEQUENCE THAT WILL BIND TO PROTEIN X
EXPRESS FUSION RNA IN CELLS
RNA-BINDING PROTEIN TO RNA Y
AAA
AAA
AAA
AAA
PROTEIN X
GENERATE CELL-FREE EXTRACT
WASH, REMOVE FROM PROTEIN X
REMOVE NEW RNA BINDING PROTEIN AND MICROSEQUENCE

FIG. 4

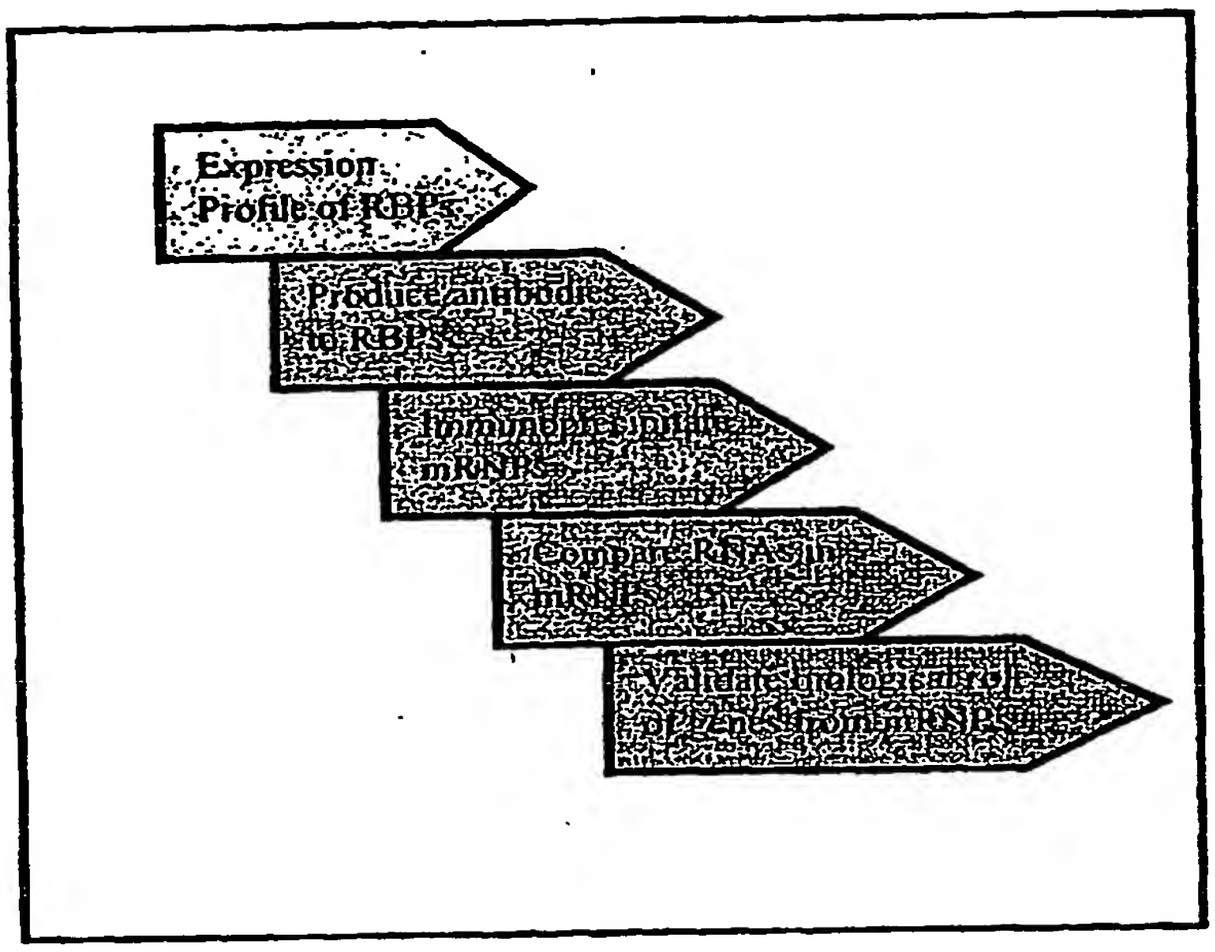

Expression Profile of RBPs
Produce antibodies to RBPs
Immunoprecipitate mRNPs
Isolate RNAs in mRNPs
Validate biological role of gene(s) in mRNP

FIG. 5

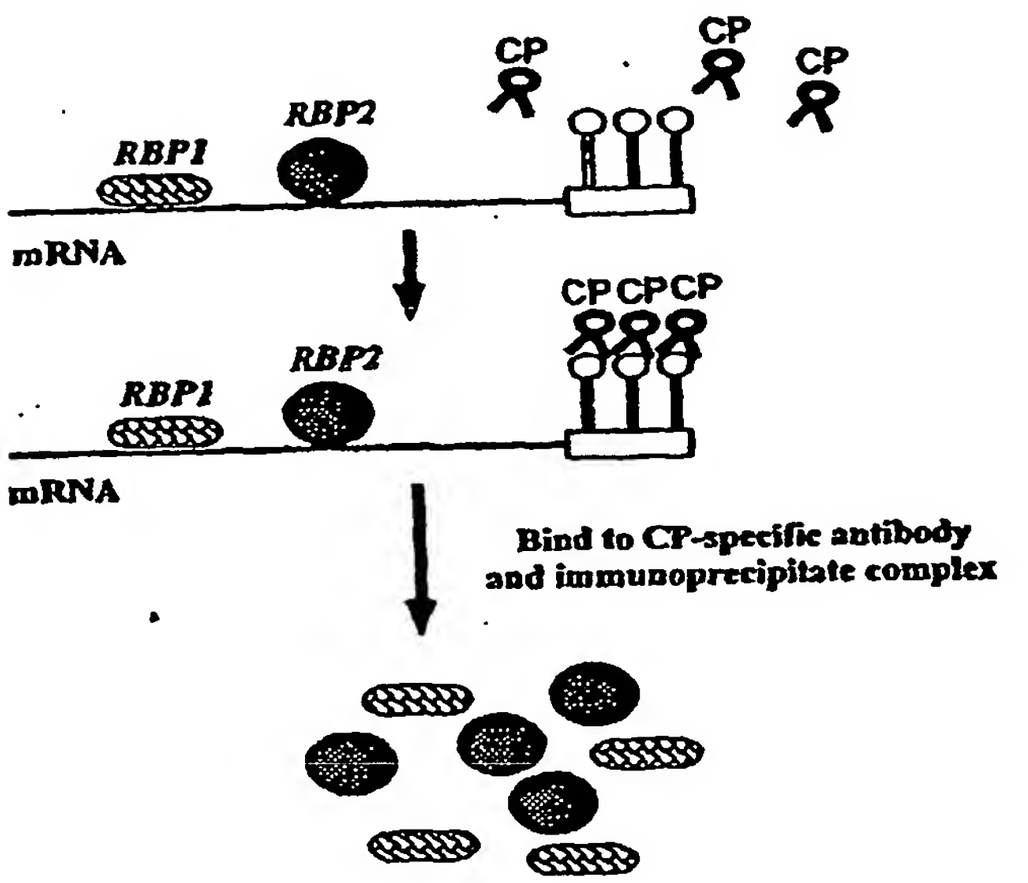

CP
CP
CP
RBP2
RBP1
mRNA
CP CP CP
RBP2
RBP1
Bind to CP-specific antibody
and immunoprecipitate complex
Purify RBPs and identify
using mass spectrometry

FIG. 6

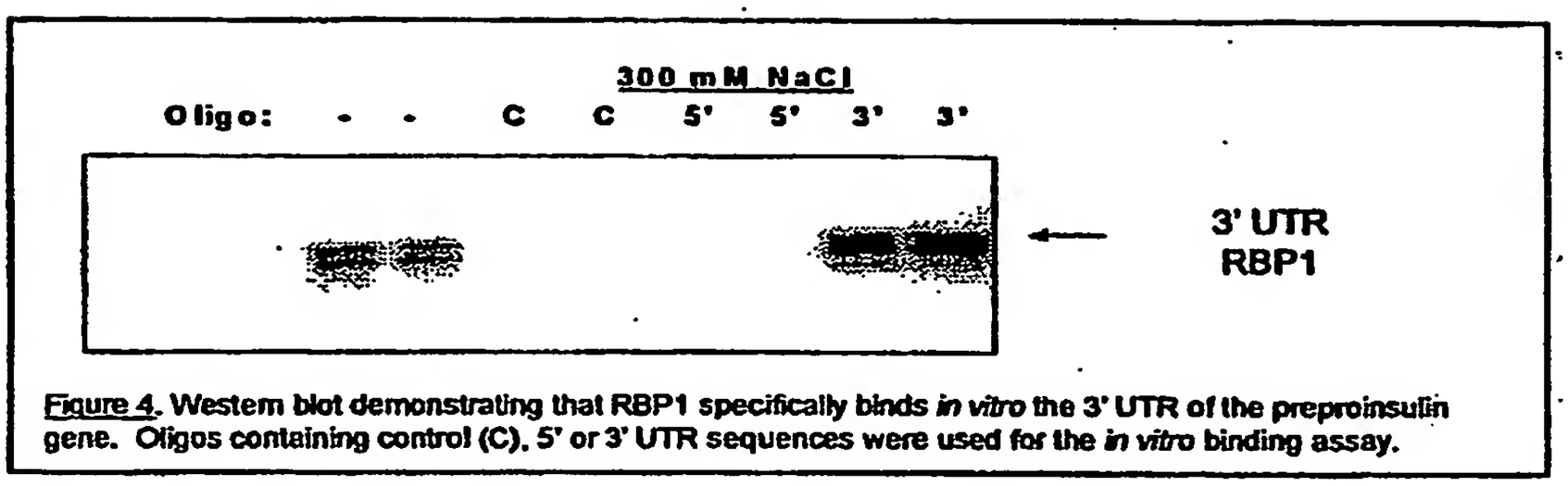
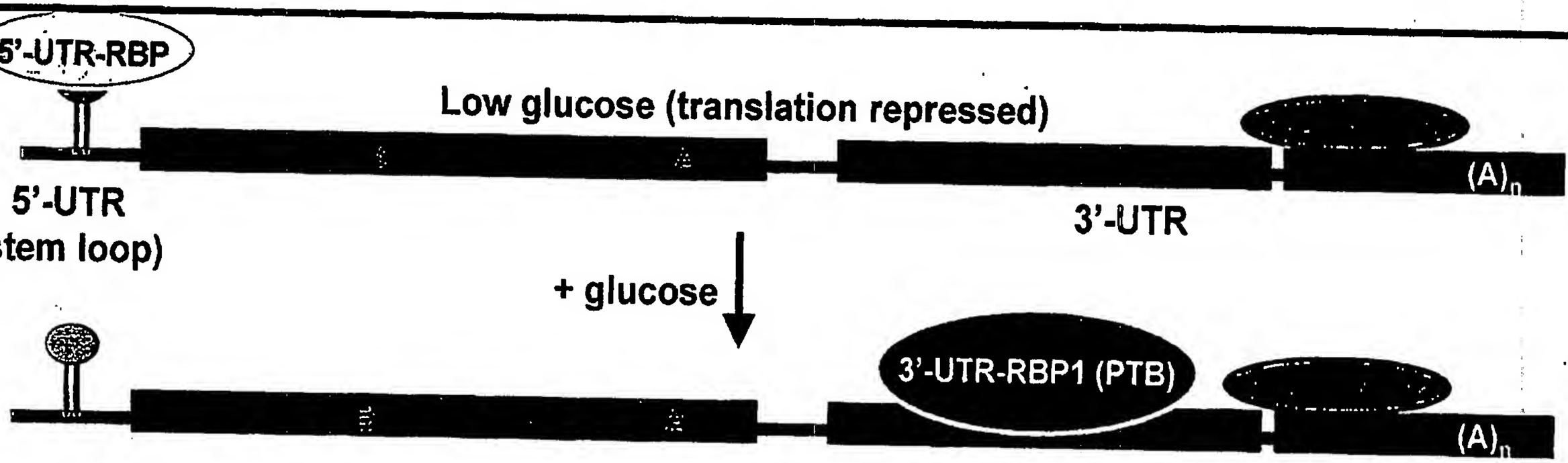


Figure 4. Western blot demonstrating that RBP1 specifically binds *in vitro* the 3' UTR of the preproinsulin gene. Oligos containing control (C), 5' or 3' UTR sequences were used for the *in vitro* binding assay.

FIG. 7

Current Paradigm of Glucose-regulated RBP-Binding to Preproinsulin mRNA



High glucose (translation active)

Model for regulation of glucose-induced preproinsulin translation by RBPs. SP, signal peptide; B,C,A, coding regions for various peptide chains of processed insulin.

FIG. 8

Title: METHODS FOR IDENTIFYING GENES INVOLVED IN GLUCOSE METABOLISM
Inventors: Cheatham et al.
Express Mail Label No. EV192307027US
Atty Docket No. RBN-003PR
Attn: Diana M. Steel
SHEET 9 OF 81

2. Target Discovery in Primary Adipocytes

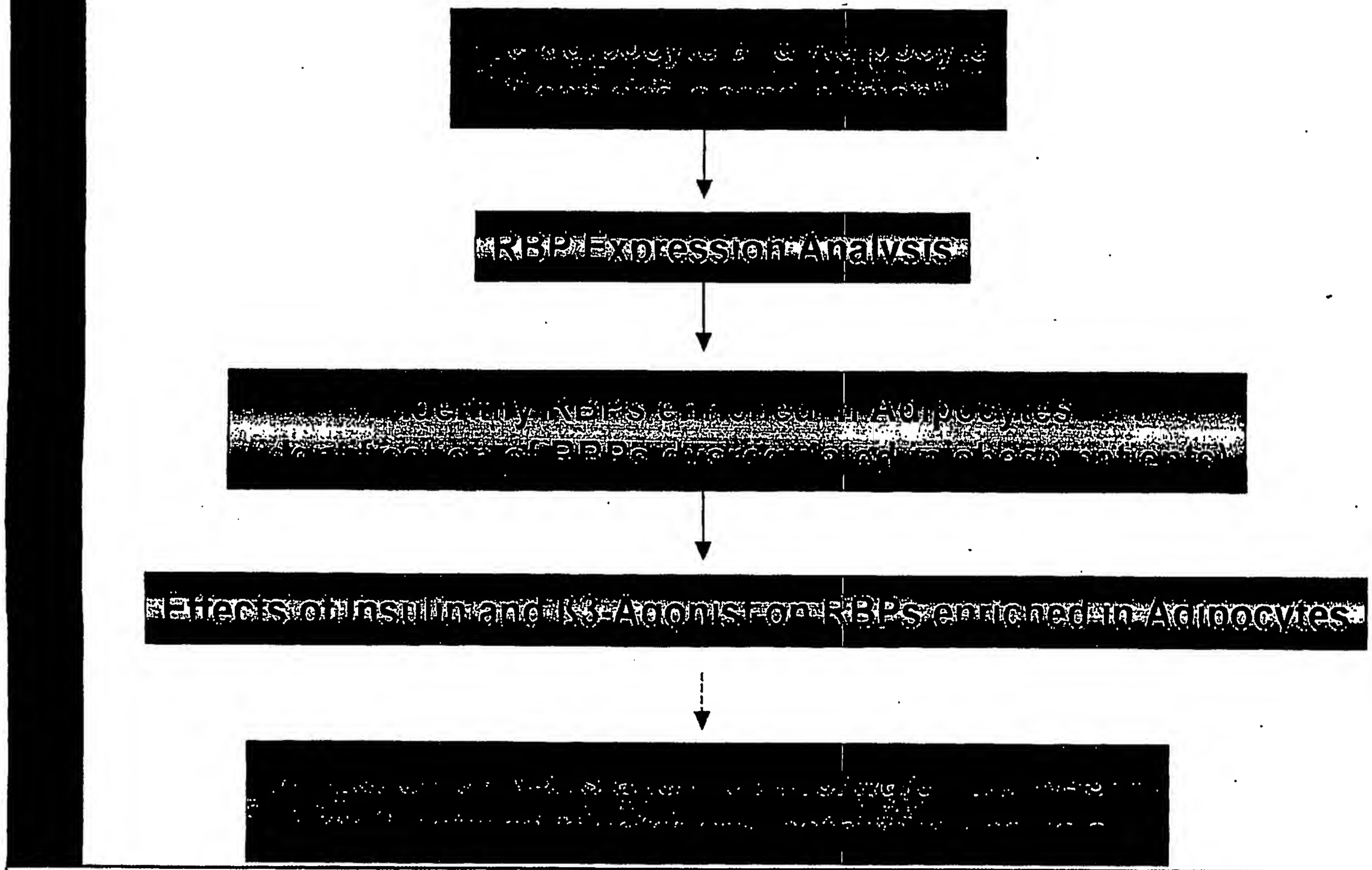
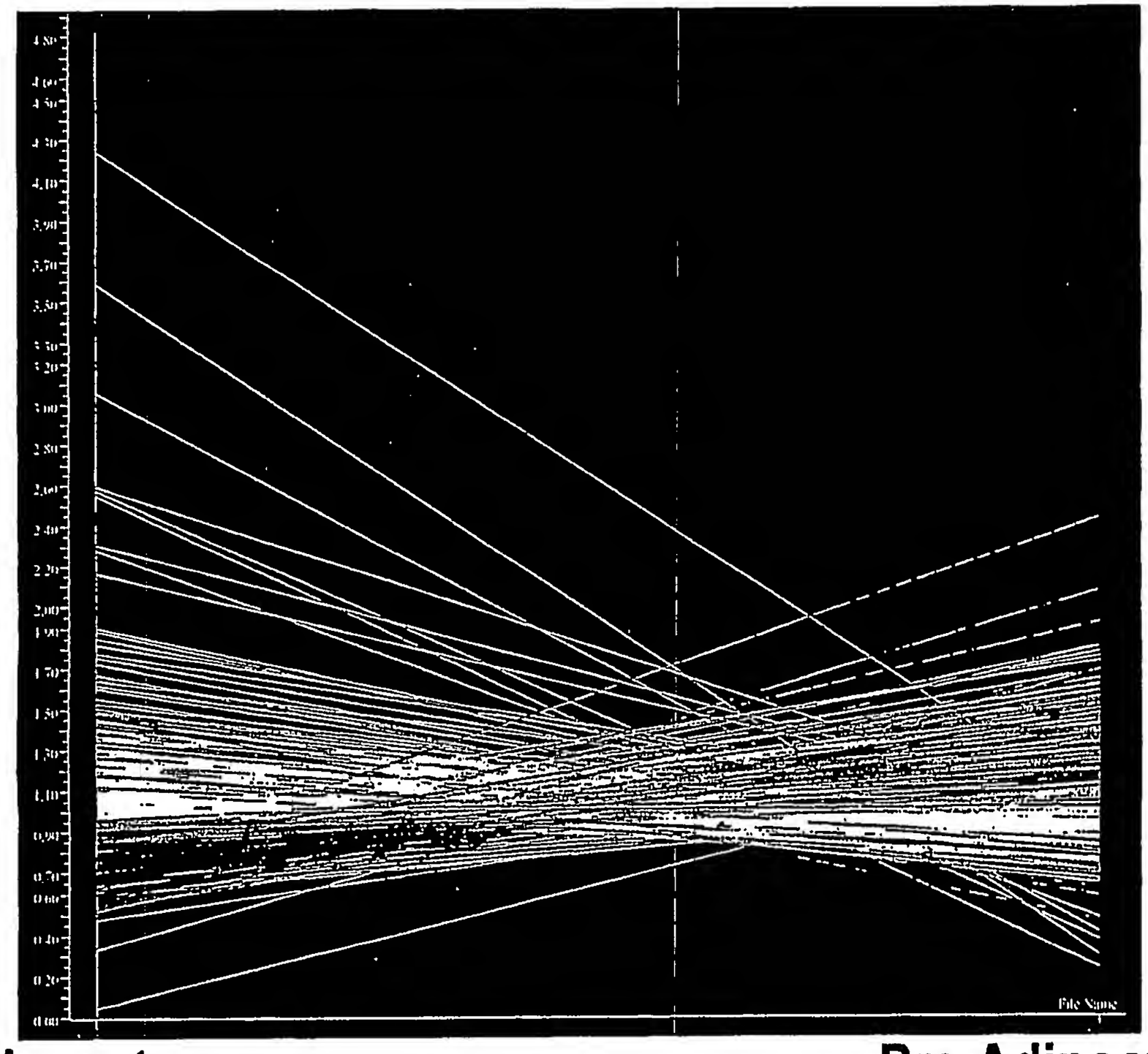


FIG. 9

Ribonomics, Inc.

Title: METHODS FOR IDENTIFYING GENES INVOLVED IN GLUCOSE METABOLISM
Inventors: Cheatham et al.
Express Mail Label No. EV192307027US
Atty Docket No. RBN-003PR
Atty: Dinna M. Steel
SHEET 10 OF 81

RBPs in Differentiating Human Adipocytes



Adipocyte

Pre-Adipocyte

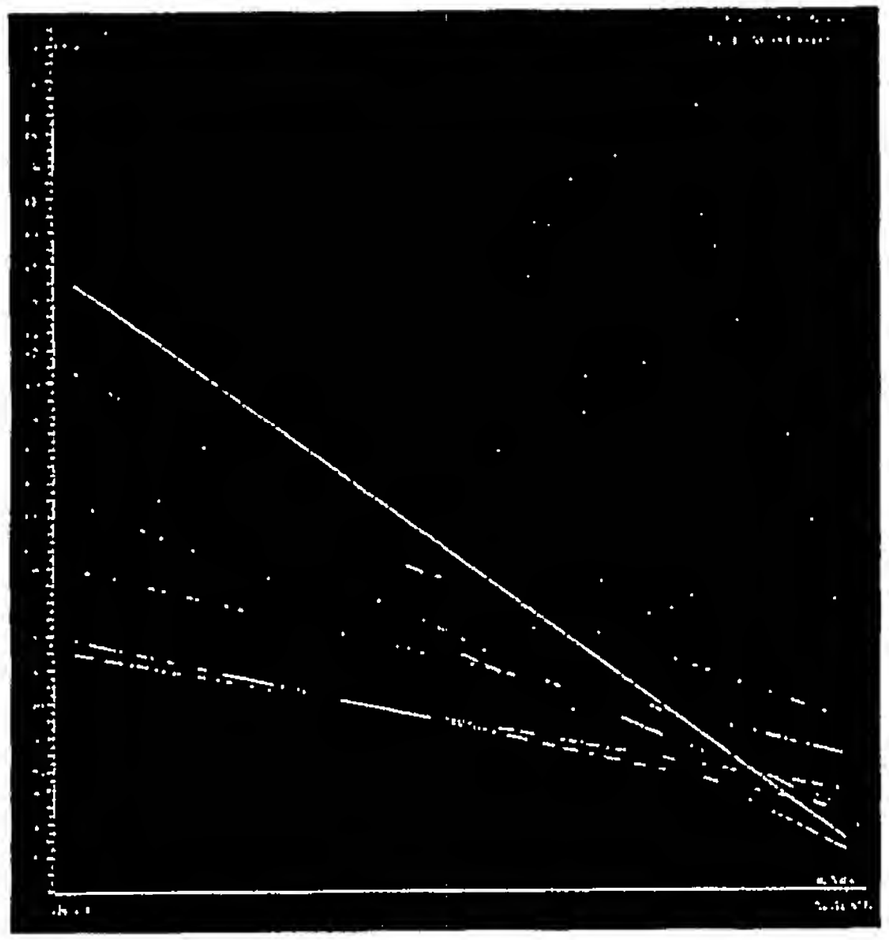
FIG. 10

Expression Data generated on human RiboChip™ microarray (1400 RBP genes)

Ribonomics, Inc.

RBPs in Differentiating Human Adipocytes

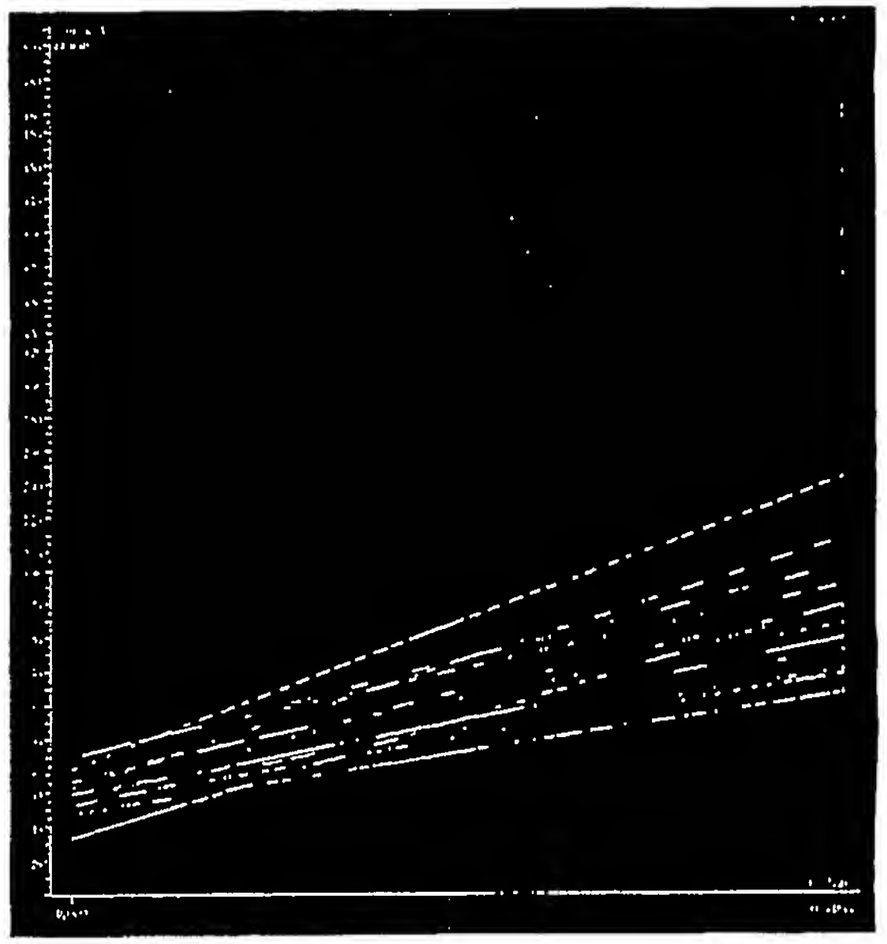
RBPs Up-regulated in Human Adipocytes



Adipocyte Pre-Adipocyte

FIG. 11A

RBPs Down-regulated in Human Adipocytes



Adipocyte Pre-Adipocyte

FIG. 11B

Ribonomics, Inc.

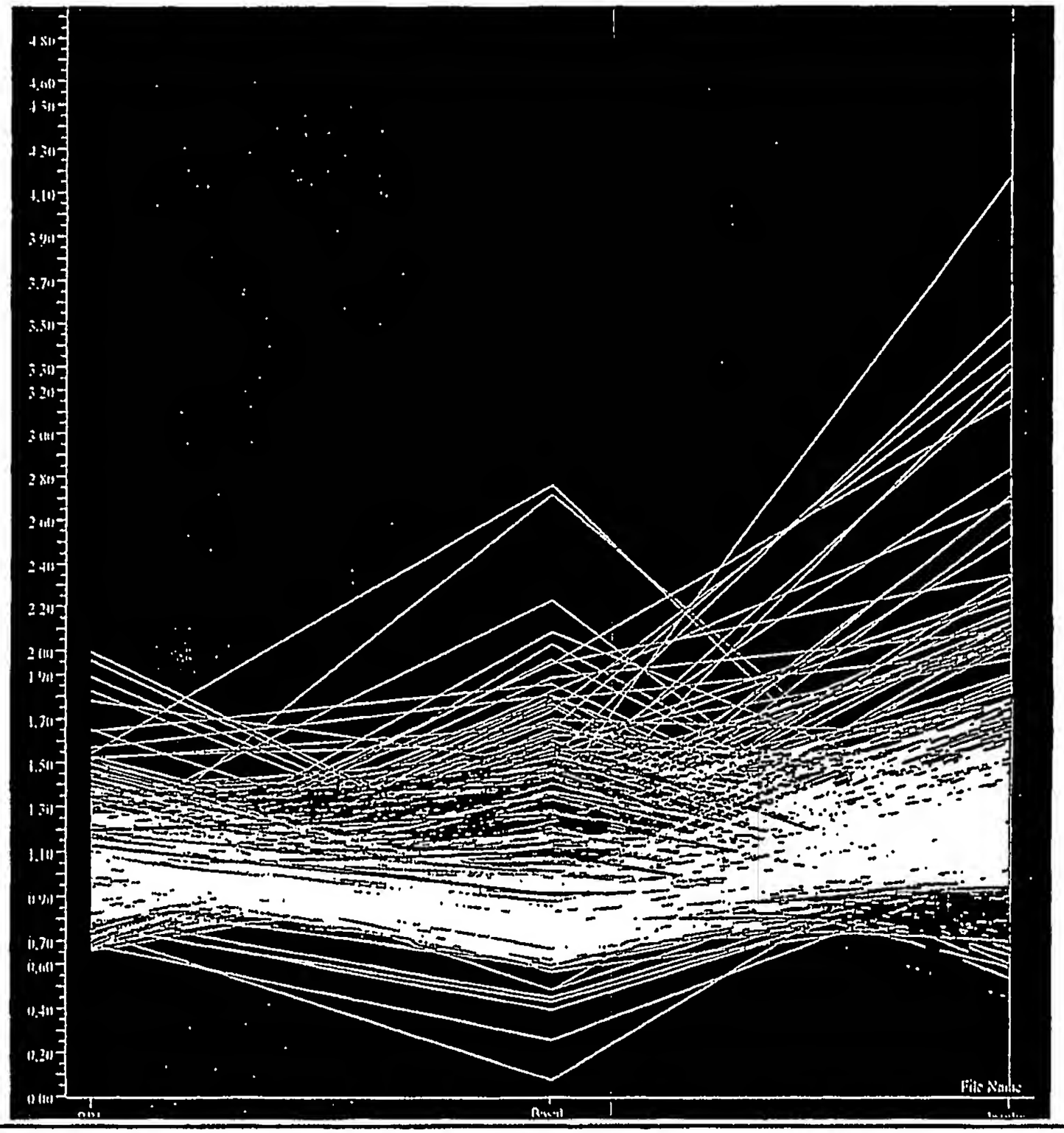
Title: METHODS FOR IDENTIFYING GENES INVOLVED IN GLUCOSE METABOLISM
Inventors: Cheatham et al.
Express Mail Label No. EV192307027US
Atty Docket No. RBN-003PR
Atty: Diana M. Steel
SHEET 12 OF 81

RBPs Up = 2x in Adipocytes vs Pre-Adipocytes from Lean Patients

GeneID	Ratio	Product	Common name
NM_006413	8.6	RPP30	ribonuclease P (30kD)
NM_020967	6.9	NCOA5	nuclear receptor coactivator 5
NM_018380	5.1	DDX28	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 28
NM_005058	4.8	RBMY1A1	RNA binding motif protein, Y chromosome, A1
XM_059828	4.5	LOC136197	similar to chromosome 22 open reading frame 5
NM_020158	3.1	RRP46	exosome component Rrp46
NM_003429	2.1	ZNF85	zinc finger protein 85 (HPF4, HTF1)
XM_068863	2.1	LOC134477	similar to CGI-79 protein
NM_000281	2.0	PCBD	pterin-4 alpha-carbinolamine dehydratase

RBP Expression in Human Adipocytes

-Effects of Insulin and the ß3 Agonist BRL-37344-



BRL-37344
1 µM

Basal

Insulin
100 nM

FIG. 13A

Ribonomics, Inc.

Title: METHODS FOR IDENTIFYING GENES INVOLVED IN GLUCOSE METABOLISM
Inventors: Cheatham et al.
Express Mail Label No. EV192307027US
Atty Docket No. RBN-003PR
Atty: Diana M. Steel
SHEET 14 OF 81

Effect of Insulin on RBPs in Adipocytes from a Lean Patient

-RBPs increased ≥1.5x up in Adipocytes-

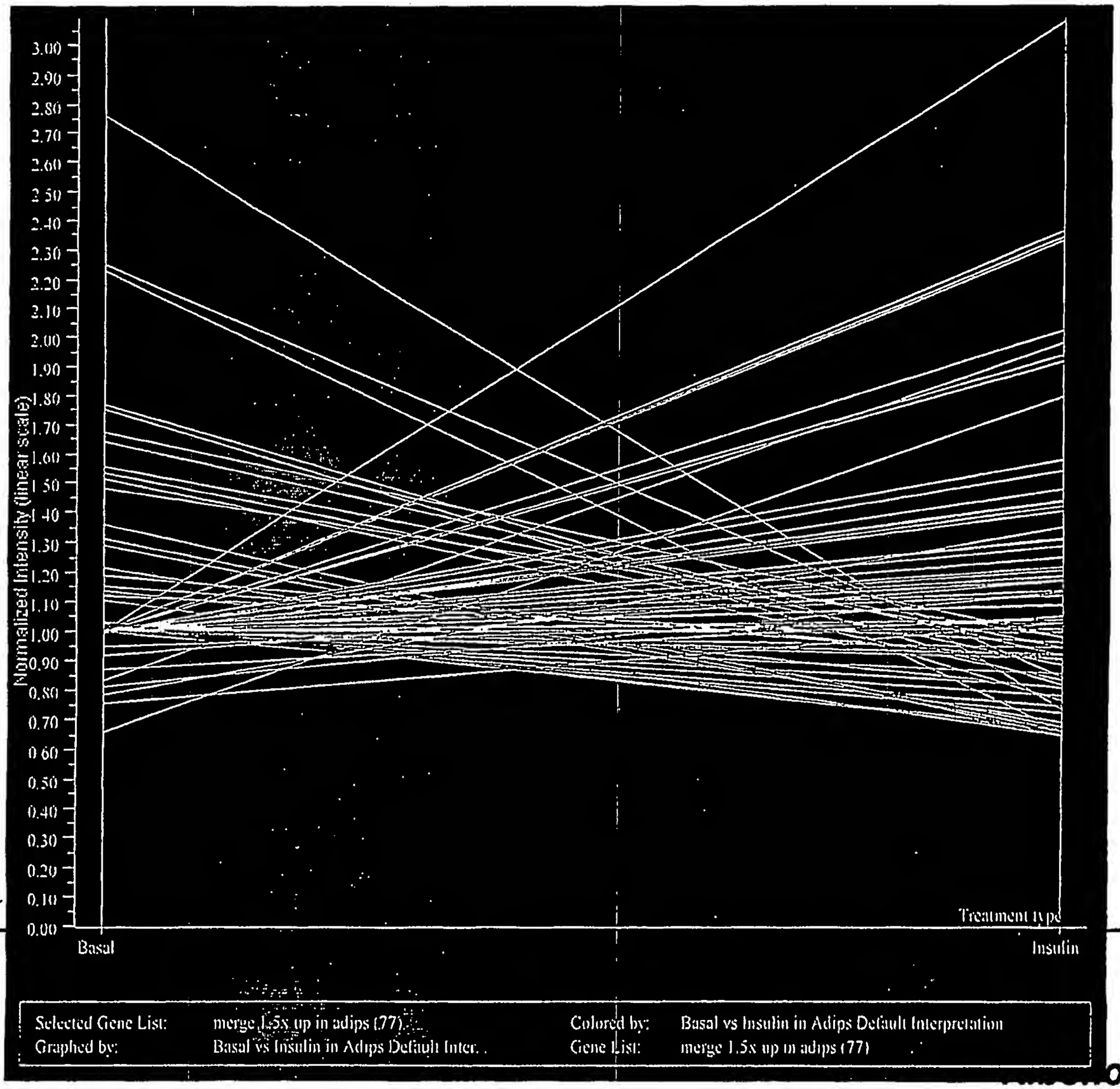


FIG. 13B

omics, Inc.

Title: METHODS FOR IDENTIFYING GENES INVOLVED IN GLUCOSE METABOLISM
Inventors: Cheatham et al.
Express Mail Label No. EV192307027US
Atty Docket No. RBN-003PR
Atty: Diana M. Steel
SHEET 15 OF 81

RBP Expression Up = 2x in Insulin-treated Adipocytes

GeneID	Ratio	Product	Common name
NM_006312	17.3	NCOR2	nuclear receptor co-repressor 2
NM_006546	3.9	IMP-1	IGF-II mRNA-binding protein 1
NM_020143	3.0	LOC56902	putatative 28 kDa protein
NM_013264	2.6	GRTH	gonadotropin-regulated testicular RNA helicase
XM_095071	2.4	LOC168400	hypothetical protein XP_095071
NM_000947	2.4	PRIM2A	primase, polypeptide 2A (58kD)
U23028	2.3	eIF-2Bepsilon	
NM_033030	2.3	BOLL	bol, boule-like (Drosophila)
XM_087251	2.3	LOC151613	similar to unnamed protein product
NM_003096	2.2	SNRPG	small nuclear ribonucleoprotein polypeptide G
NM_002502	2.2	NFKB2	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)
AF026126	2.2	HNRPD	heterogeneous nuclear ribonucleoprotein D
XM_088868	2.1	LOC163412	hypothetical protein XP_088868
NM_006209	2.1	ENPP2	ectonucleotide prophosphatase/phosphodiesterase 2
NM_001019	2.1	RPS15A	ribosomal protein S15a
XM_064989	2.0	LOC126205	similar to unnamed protein product
AF049523	2.0	HYPA	huntingtin-interacting protein HYPA/FBP11

RBPs Up = 2x in Adipocytes vs Pre-Adipocytes from Obese Patients

GeneID	Ratio	Product	Common name
XM_070830	12.9	LOC138280	similar to KIAA1138 protein
XM_092489	6.0	LOC165271	similar to hypothetical protein DKFZp434B1727 (H. sapiens)
NM_006413	5.8	RPP30	ribonuclease P (30kD)
NM_002934	4.9	RNASE2	ribonuclease, RNase A family, 2
NM_005058	4.6	RBMY1A1	RNA binding motif protein, Y chromosome, family 1, A1
NM_022360	3.8	HE3-BETA	human epididymis-specific 3 beta
XM_065361	3.6	LOC129715	similar to tudor protein
NM_020967	3.3	NCOA5	nuclear receptor coactivator 5
NM_001024	2.6	RPS21	ribosomal protein S21
NM_006187	2.3	OAS3	2'-5'oligoadenylate synthetase 3
NM_031994	2.2	RNF17	ring finger protein 17 isoform short
XM_060358	2.1	LOC127164	similar to TAR DNA binding protein
NM_002534	2.0	OAS1	2',5'-oligoadenylate synthetase 1, isoform E16
NM_018380	2.0	DDX28	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 28

Ribonomics, Inc.

GenBank ID (MWG Rat Pan 10K)	Raw Intensity Values for Total RNA Sample (Rat INS-1 cells)	Raw Intensity Values for PTB cluster sample (Rat INS-1 cells)
[AB000199]	14	56
[AB000216]	12	103
[AB000280]	11753	45813
[AB000778]	4	119
[AB000817]	110	635
[AB001453]	81	306
[AB003104]	13	95
[AB003478]	5	63
[AB003753]	123	707
[AB004276]	56	299
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[AB005052]	28	89
[AB008110]	502	2835
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[AB012235]	20	76
[AB013294]	59	180
[AB015308]	465	6615
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[AB042272]	154	812

FIG. 16A

[AB042530]	1985	31516
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[AB047915]	-1	26
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FIG. 16B

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[AF024622]	30	110
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FIG. 16C

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[AF091573]	78	253
[AF091578]	15	91
[AF091581]	-5	16
[AF093569]	9	47
[AF093625]	14	135
[AF095585]	8	29
[AF097887]	24	179
[AF100172]	283	1172
[AF100789]	19	149
[AF102262]	1101	4116
[AF102853]	17	65
[AF105329]	37	134
[AF107265]	144	852
[AF107723]	7	74
[AF108653]	17	173
[AF109393]	224	722
[AF110267]	2	64
[AF112184]	135	1646
[AF112301]	1887	8349

FIG. 16D

[AF112458]	99	438
[AF115768]	119	879
[AF118854]	9	60
[AF120109]	7	48
[AF123454]	59	1004
[AF124441]	68	290
[AF127390]	130	659
[AF130881]	825	4581
[AF135265]	42	152
[AF136584]	228	1225
[AF139918]	10	62
[AF141863]	86	264
[AF145788]	7	120
[AF147718]	76	422
[AF148797]	63	331
[AF154245]	226	1465
[AF155196]	39	376
[AF157595]	132	2543
[AF159046]	8	77
[AF159356]	47	167
[AF161588]	899	5895
[AF162914]	524	1955
[AF163318]	83	252
[AF169549]	52	171
[AF169637]	178	577
[AF171646]	24	75
[AF175209]	9303	35924
[AF175969]	20578	65455
[AF176023]	509	3330
[AF176351]	12	60
[AF177676]	39	216
[AF177683]	35	290
[AF177684]	52	366
[AF177686]	14	63
[AF177689]	31	144
[AF177693]	113	388
[AF177694]	9	210
[AF177700]	108	369
[AF178674]	73	308
[AF178975]	236	2066
[AF181992]	601	3322
[AF182037]	103	949
[AF182714]	71	354
[AF184150]	63	454
[AF184914]	213	926
[AF187100]	14	82
[AF187814]	51	590
[AF189261]	77	974
[AF192757]	166	652
[AF194029]	227	1747
[AF194442]	13	117
[AF194443]	45	187

FIG. 16E

[AF194993]	182	3154
[AF195045]	31	128
[AF196189]	8	34
[AF196202]	1148	11634
[AF196206]	10	57
[AF196215]	11	65
[AF196224]	522	8082
[AF196259]	48	435
[AF198441]	-2	6
[AF198442]	93	1813
[AF198533]	89	339
[AF199336]	224	751
[AF199337]	15	95
[AF201839]	132	505
[AF202454]	4229	23465
[AF203698]	21	70
[AF204873]	1207	7328
[AF205193]	531	2100
[AF205780]	33	197
[AF206017]	13	138
[AF207605]	534	2468
[AF208288]	6	73
[AF213506]	15	102
[AF217571]	49	277
[AF217579]	6	94
[AF218724]	55	252
[AF219629]	109	336
[AF219904]	65	334
[AF219999]	92	274
[AF220558]	150	855
[AF220761]	174	1309
[AF222337]	44	130
[AF222732]	2	24
[AF227190]	4	36
[AF230645]	247	1407
[AF236130]	1106	7079
[AF239262]	26	113
[AF239748]	104	348
[AF240765]	662	4076
[AF240766]	23	166
[AF241241]	79	851
[AF241260]	21	72
[AF241614]	74	521
[AF245040]	7	53
[AF247453]	133	975
[AF247812]	111	341
[AF248543]	62	297
[AF249748]	113	436
[AF250032]	168	798
[AF253065]	195	914
[AF254800]	23	86
[AF266164]	336	1222

FIG. 16F

[AF268030]	653	20631
[AF271033]	6	213
[AF271035]	67	580
[AF271040]	17	96
[AF271048]	-1	15
[AF271052]	104	555
[AF271054]	40	218
[AF271155]	104	344
[AF273024]	69	65461
[AF275151]	350	1021
[AF280423]	3410	15051
[AF286344]	9	71
[AF286470]	389	1798
[AF288611]	94	1242
[AF290194]	55	767
[AF290212]	970	7465
[AF292102]	120	665
[AF295404]	104	382
[AF295405]	267	2921
[AF302842]	194	807
[AF304855]	1467	7198
[AF308818]	188	1502
[AF309948]	120	729
[AF310076]	10	98
[AF314820]	49	208
[AF314960]	88	274
[AF315944]	71	331
[AF317633]	28	293
[AF318578]	356	1063
[AF321552]	1	34
[AF324255]	50	161
[AF324454]	13	46
[AF327513]	108	340
[AF327567]	138	597
[AF329091]	54	186
[AF332004]	36	205
[AF332142]	547	1645
[AF335281]	109	440
[AF335571]	32	116
[AF336857]	20	72
[AF336858]	-3	20
[AF336872]	59	1510
[AF337905]	101	304
[AF347030]	68	198
[AF347935]	694	2241
[AF347936]	347	2029
[AF348446]	12	115
[AF350373]	70	274
[AF351785]	78	329
[AF352021]	10	58
[AF352168]	14	127
[AF352169]	127	604

FIG. 16G

[AF352178]	148	542
[AF357216]	192	1027
[AF359356]	10	45
[AF361239]	298	926
[AF363960]	33	122
[AF368269]	25	110
[AF370442]	101	415
[AF370446]	2	15
[AF370882]	29	280
[AF376781]	554	22092
[AF378332]	331	1587
[AF380190]	4	117
[AF380196]	126	1665
[AF380198]	66	402
[AF386786]	7	22
[AF387102]	19	350
[AF390072]	6	49
[AF390073]	65	348
[AF393484]	153	778
[AF395897]	16	100
[AF399874]	49	253
[AF406814]	5	39
[AF412817]	109	630
[AF421368]	378	3586
[AF432872]	12	132
[AF435434]	56	857
[AF436847]	709	4585
[AF439397]	3572	10442
[AF439720]	177	901
[AF439780]	336	1508
[AF441118]	465	7115
[AF441249]	78	527
[AF442205]	25	101
[AF442357]	339	16956
[AF450298]	258	11614
[AJ000347]	53	432
[AJ000696]	0	94
[AJ001290]	27	184
[AJ001380]	150	511
[AJ001517]	44	262
[AJ001641]	605	20350
[AJ002942]	20	341
[AJ003065]	361	4786
[AJ005202]	65	558
[AJ005394]	747	7032
[AJ005396]	146	424
[AJ005424]	249	960
[AJ005776]	8	32
[AJ006070]	29	221
[AJ007485]	190	1682
[AJ010585]	51	148
[AJ011115]	14782	65476

FIG. 16H

[AJ011116]	50	146
[AJ011370]	57	1115
[AJ011432]	28	202
[AJ011811]	8	45
[AJ131111]	60	201
[AJ131902]	1084	5756
[AJ132897]	49	421
[AJ223184]	183	1728
[AJ223355]	470	3125
[AJ224431]	737	3101
[AJ224450]	30	94
[AJ224673]	855	11831
[AJ224880]	7	19
[AJ238278]	1	43
[AJ238391]	6	147
[AJ243123]	58	747
[AJ245663]	89	826
[AJ245718]	5	38
[AJ249546]	1	68
[AJ249562]	104	3482
[AJ250280]	60	427
[AJ277423]	423	17864
[AJ277448]	144	1689
[AJ277668]	67	478
[AJ277881]	88	322
[AJ277957]	51	154
[AJ286820]	6	18
[AJ291433]	120	420
[AJ294469]	20	132
[AJ295748]	198	1654
[AJ301634]	30	103
[AJ301656]	10	81
[AJ302650]	22	154
[AJ306292]	361	1694
[AJ309926]	185	1056
[AJ312933]	3	33
[AJ312934]	1135	4386
[AJ314857]	4	70
[AJ315643]	1844	6531
[AJ315657]	74	782
[AJ320522]	48	165
[AY004215]	85	305
[AY007691]	6	80
[AY008275]	59	237
[AY009158]	1451	6559
[AY011884]	105	534
[AY011941]	2	42
[AY014837]	115	347
[AY014838]	204	1290
[AY026049]	55	266
[AY026526]	62	466
[AY028418]	6	20

FIG. 16I

[AY028455]	11	49
[AY030276]	135	548
[AY032620]	2	12
[AY032665]	144	416
[AY032856]	121	400
[AY033877]	48	1227
[AY034051]	8629	65456
[AY040615]	186	923
[AY057076]	50	335
[AY059382]	92	316
[AY059383]	-3	-6
[AY063482]	35	166
[AY063759]	4578	29546
[AY064411]	105	404
[D10874]	312	1462
[D11325]	1075	5056
[D12678]	69	406
[D12978]	6757	52021
[D13555]	27	88
[D13556]	138	662
[D13963]	12	65
[D14015]	4	49
[D14046]	12	38
[D14076]	-2	89
[D14418]	5	76
[D14478]	7	146
[D14869]	154	812
[D14987]	7	23
[D16236]	48	172
[D16237]	196	907
[D16479]	2169	10142
[D17309]	87	830
[D17349]	26	147
[D17521]	1383	6785
[D17764]	10	76
[D25233]	124	498
[D25290]	20	223
[D25543]	90	862
[D26111]	632	5892
[D26112]	121	781
[D26178]	6	81
[D26307]	470	2082
[D26492]	4	31
[D26493]	22	67
[D26494]	51	398
[D26502]	20	211
[D26503]	520	7433
[D28110]	-1	48
[D28753]	1	31
[D28966]	108	321
[D31962]	37	125
[D38035]	442	2062

FIG. 16J

[D38063]	34	134
[D38067]	246	1495
[D38069]	24	237
[D45187]	144	1569
[D45241]	278	1322
[D49434]	33	172
[D50671]	102	396
[D83015]	229	1325
[D83348]	-2	46
[D83496]	90	636
[D85435]	1107	4001
[D85509]	40	138
[D86220]	19	99
[D86557]	8	51
[D86742]	2174	11776
[D87991]	43	159
[D88364]	12	71
[D88586]	943	4539
[D88666]	375	1592
[D89372]	49	172
[D89373]	136	772
[D89965]	34	165
[D90401]	103	616
[J00717]	29	995
[J00759]	182	796
[J02582]	22	81
[J02585]	-79	3480
[J02643]	33	107
[J02811]	21	154
[J02844]	5	28
[J02934]	158	459
[J02997]	56	345
[J03819]	7	39
[J04597]	44	133
[J05087]	435	1524
[J05181]	191	1268
[K02805]	5209	25596
[K02901]	101	298
[K03249]	399	2479
[L00094]	4	17
[L00981]	196	572
[L02530]	39	131
[L02842]	95	493
[L03386]	655	4368
[L03556]	126	1041
[L03557]	535	2578
[L04739]	2129	7454
[L04760]	13	78
[L05557]	23	107
[L07318]	1358	4172
[L07399]	86	385
[L07409]	124	722

FIG. 16K

[L08134]	72	703
[L08490]	474	1687
[L08497]	30	124
[L09752]	1116	10861
[L10230]	182	708
[L10336]	109	1680
[L10640]	215	3441
[L12046]	11	59
[L13202]	41	327
[L13606]	25	113
[L14446]	2258	9329
[L14610]	215	1839
[L14776]	12	65
[L14937]	173	1280
[L17318]	304	1199
[L19112]	96	461
[L20991]	78	313
[L22022]	107	539
[L22761]	11	71
[L23077]	2	14
[L25527]	49	409
[L25863]	4	22
[L25866]	17347	54268
[L27111]	759	5664
[L27129]	90	572
[L28801]	4	21
[L32601]	22	129
[L37203]	76	233
[L41045]	95	368
[L41558]	10	47
[L41686]	197	1442
[L43592]	54	203
[L46593]	37	168
[L47281]	10	34
[L48209]	-5	-7
[L75825]	30	218
[L79949]	48	270
[M11596]	170	721
[M12021]	103	462
[M12583]	50	300
[M13100]	3429	16620
[M13646]	234	1324
[M13802]	99	488
[M15527]	15	72
[M15561]	63	206
[M15562]	37	392
[M15650]	100	660
[M16409]	194	897
[M16624]	344	1101
[M16829]	847	22301
[M17092]	35	304
[M17592]	58	243

FIG. 16L

[M17703]	49	180
[M17784]	15	72
[M18330]	16	98
[M18839]	75	306
[M18840]	21	77
[M18845]	65	231
[M18847]	219	1809
[M18854]	455	1567
[M18864]	79	229
[M19042]	9	107
[M19359]	6	18
[M20297]	66	582
[M20406]	245	829
[M21817]	122	1231
[M22063]	34	208
[M22331]	74	382
[M23882]	95	410
[M23888]	6	38
[M23889]	9	120
[M25071]	10	55
[M25823]	188	3847
[M26835]	1044	5124
[M27220]	11	38
[M27440]	3305	13131
[M29853]	88	345
[M29996]	278	1524
[M30689]	191	1440
[M32973]	8	57
[M33296]	38	161
[M33648]	59	192
[M33994]	8	75
[M35086]	135	1738
[M35602]	2050	8535
[M36418]	7	21
[M37566]	-8	35
[M37568]	10	47
[M57719]	26	90
[M58040]	10	33
[M58041]	568	8047
[M59313]	296	1552
[M60388]	18	94
[M61874]	66	212
[M63482]	79	231
[M63593]	27	222
[M63837]	2	75
[M63970]	3814	26505
[M63991]	7173	36114
[M64384]	402	1991
[M64391]	39	115
[M64795]	417	2195
[M72711]	63	387
[M75168]	33	240

FIG. 16M

[M75281]	23	128
[M76537]	15	180
[M76734]	11	51
[M77183]	72	512
[M81766]	1809	11154
[M82826]	40	192
[M83210]	39	206
[M83676]	885	5487
[M83679]	53	242
[M84176]	399	4181
[M84210]	52	517
[M85301]	194	3661
[M86376]	34	131
[M86526]	663	3485
[M87785]	31	101
[M87786]	135	603
[M87787]	1557	8520
[M88356]	6	37
[M88709]	37	260
[M90661]	84	254
[M91242]	153	1429
[M91599]	40	231
[M94043]	161	891
[M95578]	2255	7160
[M96374]	420	1975
[M96377]	355	2217
[M96578]	6	29
[M96626]	62	455
[M96630]	17	84
[M97380]	29	235
[NM_012491]	5	25
[NM_012492]	24	78
[NM_012494]	75	234
[NM_012500]	11	110
[NM_012502]	47	217
[NM_012503]	69	270
[NM_012506]	395	3178
[NM_012508]	88	295
[NM_012514]	-1	40
[NM_012519]	17	87
[NM_012523]	364	7365
[NM_012524]	89	494
[NM_012528]	345	4915
[NM_012533]	50	212
[NM_012534]	86	262
[NM_012538]	48	219
[NM_012540]	495	19752
[NM_012542]	83	495
[NM_012544]	127	475
[NM_012546]	261	4118
[NM_012547]	16	79
[NM_012550]	66	228

FIG. 16N

[NM_012554]	0	23
[NM_012555]	3	26
[NM_012557]	270	1071
[NM_012561]	196	656
[NM_012563]	-2	34
[NM_012565]	341	1674
[NM_012566]	1208	10643
[NM_012568]	982	12667
[NM_012575]	239	2101
[NM_012580]	438	3023
[NM_012584]	19	132
[NM_012585]	1	34
[NM_012586]	20	58
[NM_012588]	45	207
[NM_012592]	-4	14
[NM_012603]	72	221
[NM_012605]	72	256
[NM_012608]	22	127
[NM_012609]	1303	4071
[NM_012617]	74	693
[NM_012619]	13	45
[NM_012620]	364	2154
[NM_012624]	10	61
[NM_012631]	247	1115
[NM_012632]	245	2425
[NM_012634]	54	181
[NM_012635]	134	1230
[NM_012636]	101	543
[NM_012637]	160	1186
[NM_012639]	1	43
[NM_012649]	302	7161
[NM_012650]	48	269
[NM_012651]	156	460
[NM_012654]	5	56
[NM_012656]	114	1196
[NM_012660]	76	380
[NM_012661]	175	913
[NM_012662]	185	1660
[NM_012665]	151	1160
[NM_012667]	3690	15942
[NM_012668]	89	664
[NM_012669]	92	365
[NM_012675]	152	996
[NM_012676]	1279	9998
[NM_012684]	156	591
[NM_012688]	218	872
[NM_012689]	101	568
[NM_012698]	11	69
[NM_012700]	797	2771
[NM_012702]	60	437
[NM_012705]	280	1497
[NM_012710]	34	208

FIG. 16O

[NM_012713]	1016	4602
[NM_012715]	34	152
[NM_012717]	137	467
[NM_012718]	11	34
[NM_012720]	3	19
[NM_012721]	17	85
[NM_012725]	66	229
[NM_012727]	741	4711
[NM_012734]	41	131
[NM_012735]	62	290
[NM_012736]	242	1333
[NM_012738]	1838	28990
[NM_012740]	93	312
[NM_012747]	2413	18790
[NM_012751]	56	189
[NM_012752]	49	359
[NM_012757]	22	207
[NM_012761]	5	51
[NM_012762]	7	78
[NM_012764]	31	200
[NM_012765]	19	98
[NM_012767]	75	364
[NM_012771]	11	35
[NM_012774]	366	1292
[NM_012775]	-1	19
[NM_012780]	34	300
[NM_012784]	11	55
[NM_012792]	210	1543
[NM_012793]	9	64
[NM_012803]	138	765
[NM_012805]	156	2385
[NM_012806]	47	199
[NM_012811]	215	876
[NM_012812]	19	88
[NM_012817]	67	406
[NM_012822]	446	8627
[NM_012825]	46	145
[NM_012826]	1094	3370
[NM_012827]	308	1366
[NM_012835]	33	100
[NM_012836]	1012	50551
[NM_012841]	6	26
[NM_012842]	111	366
[NM_012846]	19	181
[NM_012851]	173	590
[NM_012852]	92	671
[NM_012868]	58	226
[NM_012869]	1766	23463
[NM_012870]	8	57
[NM_012871]	14	70
[NM_012874]	206	2467
[NM_012879]	299	979

FIG. 16P

[NM_012880]	2	22
[NM_012882]	3	31
[NM_012888]	232	772
[NM_012890]	4	23
[NM_012894]	34	192
[NM_012896]	163	1400
[NM_012900]	192	647
[NM_012907]	464	2655
[NM_012908]	31	149
[NM_012910]	31	110
[NM_012914]	139	3745
[NM_012916]	19	70
[NM_012918]	3	30
[NM_012919]	1060	12499
[NM_012923]	22	133
[NM_012928]	119	857
[NM_012935]	4	19
[NM_012939]	14	43
[NM_012940]	44	443
[NM_012942]	2	44
[NM_012943]	357	1991
[NM_012944]	38	115
[NM_012948]	258	771
[NM_012949]	-5	13
[NM_012950]	175	2827
[NM_012955]	540	5152
[NM_012956]	71	239
[NM_012957]	461	3132
[NM_012958]	80	2115
[NM_012959]	231	2522
[NM_012967]	1	11
[NM_012972]	660	23540
[NM_012973]	721	3831
[NM_012975]	162	1333
[NM_012977]	58	290
[NM_012980]	16	50
[NM_012981]	-10	62
[NM_012982]	120	677
[NM_012983]	19	58
[NM_012984]	65	216
[NM_012987]	23	73
[NM_012988]	57	373
[NM_012996]	8	31
[NM_012998]	7	24
[NM_012999]	11	48
[NM_013000]	7	71
[NM_013001]	13	57
[NM_013004]	60	1160
[NM_013007]	5	35
[NM_013008]	30	155
[NM_013014]	801	3089
[NM_013021]	16	76

FIG. 16Q

[NM_013022]	78	273
[NM_013023]	8	47
[NM_013026]	616	1794
[NM_013028]	210	2486
[NM_013029]	651	4246
[NM_013031]	5	18
[NM_013033]	2	7
[NM_013034]	469	1669
[NM_013036]	2	20
[NM_013037]	100	951
[NM_013039]	7	25
[NM_013040]	13	42
[NM_013041]	25	76
[NM_013045]	3834	12036
[NM_013047]	35	117
[NM_013048]	27	151
[NM_013060]	10	115
[NM_013062]	57	180
[NM_013065]	7	27
[NM_013069]	43	240
[NM_013076]	24	98
[NM_013078]	29	104
[NM_013080]	1552	9922
[NM_013087]	1120	38041
[NM_013088]	10	61
[NM_013089]	7	29
[NM_013091]	1534	6542
[NM_013092]	50	300
[NM_013095]	89	470
[NM_013096]	134	422
[NM_013098]	905	2978
[NM_013100]	12	38
[NM_013108]	139	524
[NM_013110]	4	58
[NM_013116]	-1	30
[NM_013118]	6	25
[NM_013120]	515	3311
[NM_013122]	56	223
[NM_013123]	40	153
[NM_013124]	246	1153
[NM_013125]	339	2283
[NM_013129]	11	36
[NM_013130]	213	1001
[NM_013131]	3121	26712
[NM_013139]	18	95
[NM_013141]	27	89
[NM_013143]	455	2360
[NM_013144]	5	49
[NM_013145]	209	1744
[NM_013146]	89	263
[NM_013149]	-1	98
[NM_013153]	26	101

FIG. 16R

[NM_013155]	118	540
[NM_013163]	100	596
[NM_013166]	67	582
[NM_013167]	2	78
[NM_013170]	170	875
[NM_013173]	13	171
[NM_013178]	16	49
[NM_013179]	307	2352
[NM_013180]	5	189
[NM_013182]	61	222
[NM_013183]	54	704
[NM_013184]	11	311
[NM_013185]	6	22
[NM_013190]	58	310
[NM_013191]	241	799
[NM_013192]	1822	7711
[NM_013194]	-6	43
[NM_013195]	101	367
[NM_013196]	12	79
[NM_013197]	4	14
[NM_013200]	87	516
[NM_013214]	18	97
[NM_013217]	7	40
[NM_013218]	313	4505
[NM_013225]	19	71
[NM_013414]	6	70
[NM_016988]	34	103
[NM_016990]	10	29
[NM_016992]	2	7
[NM_016993]	234	890
[NM_016996]	2665	65450
[NM_016997]	125	984
[NM_016999]	243	1163
[NM_017001]	95	1332
[NM_017010]	6	23
[NM_017011]	218	6608
[NM_017013]	165	592
[NM_017020]	0	32
[NM_017021]	103	668
[NM_017023]	4	30
[NM_017026]	34	170
[NM_017027]	41	257
[NM_017028]	368	4551
[NM_017031]	191	614
[NM_017032]	282	1494
[NM_017034]	1	65
[NM_017036]	1281	11664
[NM_017043]	184	778
[NM_017044]	300	1164
[NM_017049]	-3	24
[NM_017053]	708	5768
[NM_017062]	1	64

FIG. 16S

[NM_017064]	12	45
[NM_017069]	43	192
[NM_017071]	1553	6481
[NM_017072]	30	96
[NM_017076]	30	88
[NM_017077]	35	365
[NM_017078]	1483	6079
[NM_017080]	31	208
[NM_017085]	3	26
[NM_017086]	26	121
[NM_017087]	14	43
[NM_017089]	12	46
[NM_017090]	0	70
[NM_017093]	105	613
[NM_017095]	23	73
[NM_017096]	8	28
[NM_017098]	2	6
[NM_017099]	519	1545
[NM_017109]	6	123
[NM_017111]	23	106
[NM_017112]	3313	22658
[NM_017123]	209	1031
[NM_017128]	29	172
[NM_017131]	5	19
[NM_017137]	15	129
[NM_017138]	7665	31134
[NM_017139]	36	212
[NM_017140]	2	14
[NM_017142]	223	5159
[NM_017145]	327	4188
[NM_017148]	45	178
[NM_017149]	908	4123
[NM_017155]	3	16
[NM_017162]	55	195
[NM_017163]	1385	14208
[NM_017164]	5	19
[NM_017170]	30	241
[NM_017172]	50	302
[NM_017174]	511	2107
[NM_017181]	9	70
[NM_017182]	0	64
[NM_017185]	20	78
[NM_017191]	89	312
[NM_017192]	49	407
[NM_017194]	0	23
[NM_017197]	287	4086
[NM_017198]	35	103
[NM_017206]	128	3825
[NM_017208]	50	158
[NM_017210]	126	581
[NM_017211]	2	22
[NM_017212]	1136	3730

FIG. 16T

[NM_017214]	294	2684
[NM_017216]	223	1889
[NM_017217]	176	1071
[NM_017218]	73	447
[NM_017224]	439	10639
[NM_017228]	110	372
[NM_017229]	32	341
[NM_017230]	121	4546
[NM_017238]	128	431
[NM_017239]	20	99
[NM_017240]	63	248
[NM_017241]	300	2067
[NM_017246]	209	4457
[NM_017251]	12	109
[NM_017253]	-1	3
[NM_017255]	744	3662
[NM_017259]	1225	16763
[NM_017260]	302	2292
[NM_017262]	195	2192
[NM_017263]	70	244
[NM_017265]	188	953
[NM_017268]	40	159
[NM_017277]	14	105
[NM_017291]	156	761
[NM_017292]	-8	4
[NM_017294]	41	282
[NM_017296]	14	47
[NM_017297]	9	44
[NM_017300]	39	204
[NM_017301]	-10	699
[NM_017303]	45	159
[NM_017312]	132	391
[NM_017315]	9278	44436
[NM_017317]	30	254
[NM_017318]	437	1277
[NM_017325]	120	415
[NM_017327]	2352	18371
[NM_017328]	4671	32449
[NM_017329]	13	50
[NM_017330]	288	1125
[NM_017332]	245	7861
[NM_017335]	-3	49
[NM_017337]	12	59
[NM_017338]	104	986
[NM_017339]	1938	11726
[NM_017344]	30	96
[NM_017347]	107	510
[NM_017348]	757	27002
[NM_017351]	22	118
[NM_017353]	368	1582
[NM_017354]	33	133
[NM_017356]	62	246

FIG. 16U

[NM_017358]	67	333
[NM_017364]	13	59
[NM_019122]	33	98
[NM_019125]	56	347
[NM_019127]	748	3555
[NM_019131]	8	39
[NM_019134]	11	45
[NM_019135]	658	2843
[NM_019136]	199	614
[NM_019137]	151	1135
[NM_019145]	188	805
[NM_019148]	26	83
[NM_019150]	106	397
[NM_019153]	99	463
[NM_019154]	545	1764
[NM_019155]	8	71
[NM_019156]	1103	3251
[NM_019159]	204	979
[NM_019162]	138	630
[NM_019168]	7	27
[NM_019174]	100	2975
[NM_019185]	5	64
[NM_019188]	6	91
[NM_019193]	106	1357
[NM_019196]	88	650
[NM_019202]	9	62
[NM_019204]	43	305
[NM_019207]	25	343
[NM_019208]	-2	18
[NM_019211]	51	172
[NM_019216]	64	271
[NM_019218]	8	55
[NM_019219]	0	41
[NM_019225]	450	1871
[NM_019229]	91	283
[NM_019237]	226	1221
[NM_019242]	4813	41682
[NM_019246]	619	24066
[NM_019248]	1275	59370
[NM_019251]	87	323
[NM_019253]	5734	19672
[NM_019258]	64	321
[NM_019261]	4	56
[NM_019262]	42	147
[NM_019270]	65	339
[NM_019271]	35	340
[NM_019272]	45	192
[NM_019274]	282	2961
[NM_019275]	7	60
[NM_019280]	94	921
[NM_019282]	84	781
[NM_019283]	104	714

FIG. 16V

[NM_019285]	333	2671
[NM_019286]	10	103
[NM_019291]	4	19
[NM_019293]	36	423
[NM_019297]	62	247
[NM_019298]	114	336
[NM_019299]	50	150
[NM_019301]	39	128
[NM_019302]	53	175
[NM_019304]	22	65
[NM_019306]	26	115
[NM_019308]	65	476
[NM_019309]	37	132
[NM_019310]	3	43
[NM_019311]	1161	16752
[NM_019312]	44	137
[NM_019313]	526	2342
[NM_019314]	702	3624
[NM_019315]	204	617
[NM_019317]	12	57
[NM_019322]	119	435
[NM_019328]	3518	40164
[NM_019329]	68	390
[NM_019335]	41	378
[NM_019339]	432	1273
[NM_019345]	38	588
[NM_019349]	25	76
[NM_019350]	266	1861
[NM_019353]	49	412
[NM_019354]	0	12
[NM_019362]	372	3492
[NM_019367]	364	4247
[NM_019371]	1	24
[NM_019374]	3774	33421
[NM_019375]	107	425
[NM_019384]	890	4239
[NM_019387]	15	93
[NM_019621]	393	2278
[NM_020072]	30	170
[NM_020076]	29	98
[NM_020077]	45	147
[NM_020078]	782	5801
[NM_020087]	3976	14046
[NM_020088]	55	281
[NM_020090]	198	3889
[NM_020095]	22	139
[NM_020099]	38	210
[NM_020100]	46	175
[NM_020101]	96	1482
[NM_020102]	10	31
[NM_020103]	138	638
[NM_020104]	48	149

FIG. 16W

[NM_020106]	11	76
[NM_020301]	4	18
[NM_020302]	2085	6690
[NM_020471]	40	203
[NM_020537]	20	187
[NM_021265]	688	3261
[NM_021266]	1328	11888
[NM_021578]	10	97
[NM_021593]	112	666
[NM_021594]	1073	5844
[NM_021596]	216	669
[NM_021654]	18	58
[NM_021657]	10	120
[NM_021661]	44	248
[NM_021664]	95	283
[NM_021666]	13	42
[NM_021669]	474	2323
[NM_021670]	151	523
[NM_021671]	5	48
[NM_021672]	8	30
[NM_021677]	57	323
[NM_021679]	6	57
[NM_021680]	-4	2107
[NM_021688]	54	313
[NM_021689]	75	312
[NM_021693]	30	250
[NM_021695]	3656	15120
[NM_021697]	19	172
[NM_021699]	26	233
[NM_021700]	51	298
[NM_021745]	106	1171
[NM_021748]	13	58
[NM_021754]	57	199
[NM_021759]	3	31
[NM_021763]	482	5631
[NM_021764]	29	97
[NM_021767]	2938	55706
[NM_021770]	154	1064
[NM_021771]	1807	19176
[NM_021772]	33	173
[NM_021775]	86	456
[NM_021837]	418	1326
[NM_021839]	102	535
[NM_021841]	3	54
[NM_021847]	48	150
[NM_021853]	931	3134
[NM_021855]	71	266
[NM_021857]	8	32
[NM_021858]	109	370
[NM_021867]	25	219
[NM_022005]	966	7070
[NM_022008]	323	5189

FIG. 16X

[NM_022175]	398	2018
[NM_022180]	215	1020
[NM_022186]	209	1279
[NM_022189]	222	1712
[NM_022190]	50	184
[NM_022194]	27	115
[NM_022204]	20	74
[NM_022205]	54	284
[NM_022206]	272	969
[NM_022210]	14	103
[NM_022211]	123	1794
[NM_022212]	313	1013
[NM_022218]	39	137
[NM_022219]	440	1499
[NM_022220]	129	6486
[NM_022222]	38	318
[NM_022223]	48	144
[NM_022227]	7	78
[NM_022236]	78	409
[NM_022239]	49	288
[NM_022240]	41	301
[NM_022244]	34	113
[NM_022247]	9	109
[NM_022264]	112	408
[NM_022266]	34	147
[NM_022271]	11	77
[NM_022272]	186	1230
[NM_022275]	69	642
[NM_022276]	111	904
[NM_022279]	279	1202
[NM_022280]	354	1323
[NM_022282]	5	30
[NM_022283]	261	5048
[NM_022287]	38	117
[NM_022292]	134	694
[NM_022293]	311	1195
[NM_022297]	8	56
[NM_022384]	34	385
[NM_022386]	405	1764
[NM_022389]	162	824
[NM_022400]	6	24
[NM_022401]	20	103
[NM_022403]	29	264
[NM_022499]	28	83
[NM_022500]	9	33
[NM_022504]	54	289
[NM_022505]	27	146
[NM_022516]	20	170
[NM_022518]	11	69
[NM_022519]	16	137
[NM_022523]	61	421
[NM_022530]	47	300

FIG. 16Y

[NM_022531]	14	113
[NM_022585]	454	5562
[NM_022586]	1036	4076
[NM_022587]	72	259
[NM_022588]	215	1668
[NM_022590]	1	29
[NM_022591]	30	163
[NM_022600]	49	1520
[NM_022602]	21	111
[NM_022603]	1	19
[NM_022604]	122	473
[NM_022611]	52	274
[NM_022612]	112	713
[NM_022617]	3346	47150
[NM_022618]	161	2104
[NM_022619]	41	801
[NM_022620]	110	952
[NM_022621]	629	4577
[NM_022622]	11	47
[NM_022627]	2291	6884
[NM_022628]	147	1196
[NM_022629]	144	656
[NM_022631]	2	86
[NM_022632]	20	80
[NM_022633]	137	1220
[NM_022635]	385	3396
[NM_022636]	53	170
[NM_022637]	185	1701
[NM_022638]	930	3334
[NM_022667]	41	248
[NM_022668]	75	288
[NM_022671]	194	1202
[NM_022673]	48	161
[NM_022678]	968	4803
[NM_022684]	58	185
[NM_022685]	41	264
[NM_022687]	131	1095
[NM_022688]	3	57
[NM_022689]	70	287
[NM_022695]	10	61
[NM_022696]	30	136
[NM_022698]	93	445
[NM_022701]	127	532
[NM_022703]	100	759
[NM_022707]	313	1959
[NM_022708]	5	16
[NM_022709]	1210	4183
[NM_022710]	31	253
[NM_022711]	249	3059
[NM_022714]	8	51
[NM_022799]	11866	65480
[NM_022848]	88	297

FIG. 16Z

[NM_022852]	37	135
[NM_022853]	125	390
[NM_022856]	88	710
[NM_022857]	64	344
[NM_022860]	70	464
[NM_022861]	64	192
[NM_022862]	5500	25149
[NM_022864]	2	112
[NM_022865]	40	119
[NM_022866]	3253	15310
[NM_022930]	-45	52
[NM_022933]	3	67
[NM_022935]	15	134
[NM_022937]	868	2854
[NM_022938]	28	153
[NM_022941]	5	84
[NM_022942]	134	538
[NM_022946]	45	136
[NM_022950]	24	92
[NM_022951]	15	74
[NM_022953]	5	53
[NM_022955]	557	2729
[NM_022956]	1012	7371
[NM_022957]	59	174
[NM_022961]	36	137
[NM_023021]	257	888
[NM_023023]	223	2314
[NM_023024]	3	72
[NM_023025]	9	51
[NM_023026]	93	305
[NM_023099]	29	259
[NM_023103]	94	880
[NM_023950]	58	304
[NM_023951]	9	30
[NM_023952]	275	940
[NM_023956]	242	741
[NM_023968]	984	5123
[NM_023970]	6	50
[NM_023973]	12	65
[NM_023974]	28	85
[NM_023976]	33	143
[NM_023981]	30	200
[NM_023982]	398	2499
[NM_023983]	68	244
[NM_023987]	101	367
[NM_023988]	402	4304
[NM_023990]	27	101
[NM_023991]	55	198
[NM_023992]	8	63
[NM_023996]	4	20
[NM_023998]	19	84
[NM_023999]	5	29

FIG. 16AA

[NM_024130]	2	52
[NM_024132]	570	4930
[NM_024137]	41	693
[NM_024139]	24	189
[NM_024140]	45	383
[NM_024141]	105	371
[NM_024144]	192	827
[NM_024146]	118	1329
[NM_024150]	1	4
[NM_024153]	39	168
[NM_024154]	93	664
[NM_024157]	2869	16402
[NM_024159]	63	185
[NM_024163]	169	829
[NM_024349]	81	563
[NM_024353]	3598	17202
[NM_024354]	108	420
[NM_024355]	24	79
[NM_024358]	148	1386
[NM_024366]	129	759
[NM_024368]	7	131
[NM_024372]	331	2134
[NM_024375]	9	29
[NM_024380]	55	392
[NM_024381]	162	503
[NM_024383]	60	278
[NM_024391]	30	97
[NM_024397]	27	651
[NM_024400]	223	9299
[NM_024401]	154	643
[NM_024489]	37	141
[NM_030585]	1	16
[NM_030827]	68	342
[NM_030830]	103	467
[NM_030831]	39	382
[NM_030837]	680	31255
[NM_030838]	1365	17114
[NM_030840]	146	491
[NM_030845]	73	239
[NM_030848]	16	65
[NM_030849]	121	440
[NM_030854]	108	561
[NM_030858]	95	314
[NM_030860]	60	246
[NM_030862]	68	659
[NM_030863]	179	635
[NM_030869]	1364	5338
[NM_030871]	109	894
[NM_030872]	36	237
[NM_030987]	93	290
[NM_030988]	6	24
[NM_030989]	2	12

FIG. 16AB

[NM_030990]	576	4847
[NM_030991]	451	2446
[NM_030995]	730	2510
[NM_030997]	16	91
[NM_030999]	9	90
[NM_031006]	17	85
[NM_031007]	249	2654
[NM_031010]	-3	26
[NM_031012]	74	284
[NM_031013]	219	1290
[NM_031024]	629	1851
[NM_031027]	929	6140
[NM_031031]	1	31
[NM_031033]	42	165
[NM_031035]	291	1078
[NM_031036]	24	79
[NM_031039]	12	114
[NM_031044]	306	1182
[NM_031047]	24	73
[NM_031050]	108	713
[NM_031057]	2325	9564
[NM_031073]	103	614
[NM_031074]	195	2163
[NM_031075]	57	384
[NM_031079]	21	99
[NM_031081]	101	1197
[NM_031083]	5	71
[NM_031087]	117	678
[NM_031088]	11	57
[NM_031094]	31	99
[NM_031095]	-1	4
[NM_031098]	189	560
[NM_031107]	19	183
[NM_031115]	111	365
[NM_031116]	42	180
[NM_031124]	8	22
[NM_031127]	1451	5224
[NM_031130]	85	600
[NM_031132]	14	198
[NM_031133]	952	3794
[NM_031135]	15	67
[NM_031136]	2	14
[NM_031139]	14	165
[NM_031141]	953	6965
[NM_031142]	9	79
[NM_031143]	231	833
[NM_031144]	23	101
[NM_031152]	8	32
[NM_031235]	102	366
[NM_031238]	3	10
[NM_031241]	621	3501
[NM_031242]	29	89

FIG. 16AC

[NM_031316]	194	17470
[NM_031321]	47	141
[NM_031327]	16	108
[NM_031328]	43	160
[NM_031332]	149	500
[NM_031334]	243	1486
[NM_031342]	141	1054
[NM_031343]	2	14
[NM_031347]	3	20
[NM_031348]	27	96
[NM_031353]	20	139
[NM_031357]	485	7053
[NM_031358]	29	354
[NM_031359]	88	926
[NM_031502]	32	184
[NM_031503]	44	584
[NM_031511]	59	216
[NM_031512]	17	55
[NM_031513]	16	50
[NM_031518]	312	5077
[NM_031521]	449	1684
[NM_031528]	20	593
[NM_031530]	314	3313
[NM_031534]	3191	11630
[NM_031535]	119	569
[NM_031536]	20	74
[NM_031538]	94	1080
[NM_031539]	1722	5203
[NM_031541]	2	14
[NM_031548]	11333	62033
[NM_031549]	286	1420
[NM_031550]	48	296
[NM_031552]	9	30
[NM_031558]	2093	19823
[NM_031561]	138	892
[NM_031564]	30	239
[NM_031569]	21	187
[NM_031577]	969	3186
[NM_031581]	445	5820
[NM_031586]	1663	25218
[NM_031588]	337	1251
[NM_031593]	536	2152
[NM_031597]	26	79
[NM_031598]	6	22
[NM_031600]	25	110
[NM_031605]	523	3279
[NM_031609]	230	858
[NM_031612]	147	558
[NM_031614]	263	1171
[NM_031615]	573	1853
[NM_031616]	-3	17
[NM_031617]	163	1158

FIG. 16AD

[NM_031627]	26	86
[NM_031628]	1122	9033
[NM_031631]	872	5498
[NM_031633]	264	3904
[NM_031634]	443	1574
[NM_031635]	528	4489
[NM_031642]	1081	4611
[NM_031644]	139	1408
[NM_031646]	5	20
[NM_031647]	48	206
[NM_031648]	31	182
[NM_031652]	3	56
[NM_031653]	15	85
[NM_031657]	11	77
[NM_031658]	32	202
[NM_031659]	8	44
[NM_031663]	1	13
[NM_031665]	54	720
[NM_031669]	113	385
[NM_031675]	3	60
[NM_031677]	121	473
[NM_031678]	25	89
[NM_031684]	87	342
[NM_031694]	139	442
[NM_031698]	7	21
[NM_031699]	752	2268
[NM_031700]	31	153
[NM_031704]	618	2220
[NM_031713]	56	163
[NM_031716]	35	118
[NM_031720]	56	337
[NM_031721]	33	120
[NM_031725]	1530	4680
[NM_031727]	29	91
[NM_031728]	21	64
[NM_031736]	30	91
[NM_031738]	188	1424
[NM_031741]	77	492
[NM_031742]	26	187
[NM_031746]	46	475
[NM_031747]	2229	17309
[NM_031749]	379	2231
[NM_031751]	4765	40733
[NM_031756]	315	2052
[NM_031758]	104	471
[NM_031762]	-1	19
[NM_031766]	41	169
[NM_031778]	44	278
[NM_031779]	30	142
[NM_031783]	6	54
[NM_031785]	276	3012
[NM_031788]	-5	19

FIG. 16AE

[NM_031790]	220	2732
[NM_031792]	113	455
[NM_031796]	129	375
[NM_031797]	10	64
[NM_031798]	723	2301
[NM_031800]	24	73
[NM_031801]	185	806
[NM_031802]	72	313
[NM_031803]	378	1374
[NM_031806]	107	381
[NM_031808]	141	721
[NM_031809]	32	126
[NM_031813]	6	18
[NM_031817]	3296	23110
[NM_031818]	422	1326
[NM_031819]	1	17
[NM_031820]	48	1736
[NM_031822]	24	160
[NM_031823]	24	249
[NM_031826]	12	40
[NM_031828]	725	4192
[NM_031830]	138	835
[NM_031831]	-3	18
[NM_031834]	73	256
[NM_031835]	8	39
[NM_031967]	314	1914
[NM_031972]	9	27
[NM_031973]	2	100
[NM_031976]	9	39
[NM_031980]	306	2145
[NM_031982]	54	198
[NM_031986]	59	430
[NM_032060]	92	531
[NM_032061]	219	883
[NM_032065]	144	1612
[NM_032073]	16	45
[NM_032074]	17	98
[NM_032080]	303	1212
[NM_032081]	34	195
[NM_032084]	155	582
[NM_032462]	178	529
[NM_032617]	68	921
[NM_032990]	51	207
[NM_033095]	69	474
[NM_033233]	153	1018
[NM_033352]	9	69
[NM_033359]	49	319
[NM_033376]	6	52
[NM_033441]	231	718
[NM_033442]	886	6606
[NM_033499]	2	32
[NM_052799]	19	286

FIG. 16AF

[NM_052805]	1444	8183
[NM_052806]	42	142
[NM_052807]	2794	13151
[NM_052809]	64	224
[NM_052979]	6	18
[NM_052980]	530	2454
[NM_052983]	6259	21704
[NM_053021]	30	113
[NM_053288]	108	369
[NM_053296]	211	2440
[NM_053302]	153	740
[NM_053314]	0	39
[NM_053317]	453	1527
[NM_053318]	11311	56268
[NM_053321]	121	467
[NM_053322]	61	400
[NM_053324]	25	78
[NM_053325]	473	7228
[NM_053328]	46	270
[NM_053329]	16	64
[NM_053332]	2220	65475
[NM_053333]	1022	4004
[NM_053335]	54	595
[NM_053338]	1026	3117
[NM_053339]	8	89
[NM_053343]	5	20
[NM_053346]	46	209
[NM_053348]	10	86
[NM_053349]	21	91
[NM_053350]	33	148
[NM_053351]	45	350
[NM_053353]	67	825
[NM_053356]	312	1211
[NM_053357]	130	385
[NM_053364]	211	814
[NM_053369]	135	694
[NM_053372]	239	798
[NM_053373]	42	172
[NM_053379]	4	19
[NM_053390]	2818	9482
[NM_053395]	0	1
[NM_053400]	21	111
[NM_053402]	114	649
[NM_053407]	89	1838
[NM_053410]	11	57
[NM_053415]	101	708
[NM_053421]	62	196
[NM_053424]	346	5311
[NM_053429]	5	23
[NM_053433]	1081	3547
[NM_053434]	1	14
[NM_053436]	92	1094

FIG. 16AG

[NM_053437]	221	1085
[NM_053438]	2	24
[NM_053457]	246	1625
[NM_053463]	31	147
[NM_053465]	52	296
[NM_053469]	1819	5650
[NM_053472]	24	311
[NM_053473]	80	401
[NM_053476]	969	9113
[NM_053481]	17	125
[NM_053486]	2	16
[NM_053491]	30	180
[NM_053497]	710	5607
[NM_053500]	5	36
[NM_053502]	139	967
[NM_053516]	-3	21
[NM_053520]	6	80
[NM_053521]	525	2240
[NM_053524]	75	3030
[NM_053527]	222	713
[NM_053529]	324	4868
[NM_053534]	52	419
[NM_053536]	36	395
[NM_053541]	3	59
[NM_053543]	2	27
[NM_053544]	15	68
[NM_053548]	29	136
[NM_053550]	85	411
[NM_053553]	40	272
[NM_053576]	12	87
[NM_053578]	1104	3757
[NM_053583]	3	31
[NM_053584]	21	95
[NM_053585]	525	8197
[NM_053589]	239	755
[NM_053594]	25	102
[NM_053595]	5450	20696
[NM_053601]	14	69
[NM_053604]	42	858
[NM_053609]	200	2805
[NM_053622]	40	183
[NM_053623]	3	39
[NM_053624]	65	284
[NM_053626]	361	2227
[NM_053629]	1171	4214
[NM_053639]	17	62
[NM_053640]	101	575
[NM_053641]	696	6076
[NM_053643]	34	100
[NM_053645]	6	32
[NM_053646]	100	338
[NM_053648]	5	19

FIG. 16AH

[NM_053649]	225	992
[NM_053655]	13	103
[NM_053665]	39	206
[NM_053666]	321	1476
[NM_053667]	12	64
[NM_053669]	265	802
[NM_053675]	10	304
[NM_053678]	65	387
[NM_053683]	3195	12724
[NM_053686]	60	291
[NM_053687]	6	23
[NM_053689]	53	256
[NM_053699]	1162	4187
[NM_053700]	57	187
[NM_053703]	26	95
[NM_053706]	26	124
[NM_053707]	73	279
[NM_053713]	108	505
[NM_053714]	52	505
[NM_053721]	7	54
[NM_053722]	98	466
[NM_053724]	54	249
[NM_053726]	20	157
[NM_053727]	32	133
[NM_053729]	136	937
[NM_053733]	38	168
[NM_053735]	31	537
[NM_053737]	13	63
[NM_053738]	14	43
[NM_053741]	24	177
[NM_053751]	52	172
[NM_053753]	504	2015
[NM_053754]	49	703
[NM_053758]	73	386
[NM_053763]	99	645
[NM_053764]	1447	4977
[NM_053765]	-7	30
[NM_053769]	5	74
[NM_053770]	33	304
[NM_053772]	31	121
[NM_053774]	70	226
[NM_053777]	21	98
[NM_053778]	164	1594
[NM_053804]	45	149
[NM_053806]	178	969
[NM_053808]	764	6243
[NM_053816]	68	223
[NM_053817]	184	1440
[NM_053819]	174	1282
[NM_053820]	48	359
[NM_053821]	5	30
[NM_053830]	108	573

FIG. 16AI

[NM_053832]	39	339
[NM_053834]	35	246
[NM_053836]	6	75
[NM_053844]	22	89
[NM_053847]	42	243
[NM_053848]	3645	18448
[NM_053854]	25	94
[NM_053863]	31	99
[NM_053865]	761	6659
[NM_053868]	437	5644
[NM_053869]	16	261
[NM_053870]	117	621
[NM_053879]	53	307
[NM_053883]	1420	6704
[NM_053885]	31	94
[NM_053887]	8815	43317
[NM_053891]	126	875
[NM_053893]	1085	3340
[NM_053895]	155	18508
[NM_053896]	17	99
[NM_053897]	62	779
[NM_053902]	95	327
[NM_053903]	79	372
[NM_053907]	4	47
[NM_053910]	27	176
[NM_053919]	61	447
[NM_053920]	-2	5
[NM_053921]	38	243
[NM_053922]	32	99
[NM_053924]	19	113
[NM_053926]	21	211
[NM_053930]	45	279
[NM_053949]	16	119
[NM_053951]	9	52
[NM_053952]	35	102
[NM_053957]	252	1111
[NM_053958]	10	314
[NM_053959]	-2	13
[NM_053964]	30	92
[NM_053967]	328	1700
[NM_053977]	338	1681
[NM_053984]	39	146
[NM_053988]	24	87
[NM_053989]	21	196
[NM_053992]	-6	3143
[NM_053994]	166	3782
[NM_053998]	32	106
[NM_054000]	629	3643
[NM_054002]	2	13
[NM_054005]	689	2210
[NM_054007]	2807	18407
[NM_054008]	97	446

FIG. 16AJ

[NM_054009]	-2	47
[NM_054011]	118	774
[NM_057100]	47	153
[NM_057101]	145	724
[NM_057103]	202	9437
[NM_057109]	765	3251
[NM_057115]	1459	5368
[NM_057116]	125	845
[NM_057118]	91	611
[NM_057121]	242	909
[NM_057124]	26	94
[NM_057125]	55	302
[NM_057135]	18	108
[NM_057136]	1185	7299
[NM_057137]	4	55
[NM_057144]	2284	9582
[NM_057146]	491	1431
[NM_057147]	372	1851
[NM_057148]	11933	46277
[NM_057149]	13	58
[NM_057151]	7	56
[NM_057156]	9643	45085
[NM_057184]	76	541
[NM_057186]	64	564
[NM_057187]	38	397
[NM_057196]	-3	37
[NM_057201]	38	119
[NM_057209]	77	402
[NM_057210]	466	1415
[NM_057211]	150	592
[NM_057212]	186	1002
[NM_057213]	19	168
[NM_058208]	176	1280
[NM_078619]	43	159
[NM_078621]	2	94
[NM_078622]	9	62
[NM_080399]	34	393
[NM_080400]	1	65
[NM_080410]	88	967
[NM_080411]	348	1097
[NM_080477]	27	139
[NM_080582]	24	268
[NM_080584]	-716	1127
[NM_080586]	25	85
[NM_080587]	2	32
[NM_080688]	16	48
[NM_080689]	21	187
[NM_080690]	26	238
[RATTUS00010]	5	52
[RATTUS00014]	172	876
[RATTUS00022]	8	37
[RATTUS00030]	259	1198

FIG. 16AK

[RATTUS00032]	259	778
[RATTUS00039]	15	45
[RATTUS00045]	70	353
[RATTUS00049]	167	870
[RATTUS00056]	39	205
[RATTUS00059]	553	3505
[RATTUS00060]	4	21
[RATTUS00061]	93	452
[RATTUS00069]	8	32
[RATTUS00077]	75	439
[RATTUS00082]	25	104
[RATTUS00085]	44	1166
[RATTUS00091]	1037	3599
[RATTUS00092]	102	706
[RATTUS00094]	10	44
[RATTUS00116]	13	59
[RATTUS00120]	73	315
[RATTUS00122]	12	49
[RATTUS00142]	52	156
[RATTUS00144]	574	8802
[RATTUS00148]	39	177
[RATTUS00156]	340	2217
[RATTUS00172]	7	53
[RATTUS00190]	114	646
[RATTUS00192]	2431	11232
[RATTUS00193]	445	1970
[RATTUS00203]	34	99
[RATTUS00204]	105	615
[RATTUS00205]	461	7568
[RATTUS00218]	89	778
[RATTUS00221]	16	48
[RATTUS00223]	1287	5979
[RATTUS00224]	87	1089
[RATTUS00225]	14	71
[RATTUS00226]	177	889
[RATTUS00231]	32	105
[RATTUS00240]	31	134
[RATTUS00253]	191	746
[RATTUS00264]	857	7105
[RATTUS00276]	1656	14679
[RATTUS00277]	10	216
[RATTUS00284]	92	268
[RATTUS00286]	19	89
[RATTUS00287]	102	1770
[RATTUS00292]	1919	10400
[RATTUS00293]	59	222
[RATTUS00309]	3	13
[RATTUS00311]	114	1338
[RATTUS00314]	31	111
[RATTUS00315]	13	75
[RATTUS00316]	92	274
[RATTUS00321]	9	59

FIG. 16AL

[RATTUS00330]	61	190
[RATTUS00348]	605	2166
[RATTUS00367]	58	488
[RATTUS00375]	56	289
[RATTUS00378]	414	4019
[RATTUS00379]	1514	7544
[RATTUS00380]	8	77
[RATTUS00390]	48	197
[RATTUS00391]	31	94
[RATTUS00398]	201	755
[RATTUS00400]	32	128
[RATTUS00404]	-1	8
[RATTUS00408]	32	186
[RATTUS00410]	2538	11014
[RATTUS00419]	10	59
[RATTUS00433]	133	922
[RATTUS00434]	834	7333
[RATTUS00437]	29	117
[RATTUS00438]	484	4616
[RATTUS00446]	0	-3
[RATTUS00451]	1472	7443
[RATTUS00454]	107	1167
[RATTUS00470]	8	83
[RATTUS00472]	40	245
[RATTUS00485]	56	293
[RATTUS00487]	74	335
[RATTUS00495]	74	458
[RATTUS00501]	68	239
[RATTUS00503]	66	191
[RATTUS00509]	-7	21
[RATTUS00510]	84	1590
[RATTUS00512]	3	30
[RATTUS00513]	195	575
[RATTUS00517]	9	53
[RATTUS00522]	57	261
[RATTUS00524]	15	76
[RATTUS00525]	288	6222
[RATTUS00526]	99	309
[RATTUS00528]	2568	8958
[RATTUS00529]	525	3110
[RATTUS00530]	9	157
[RATTUS00536]	7	34
[RATTUS00539]	2	24
[RATTUS00540]	44	325
[RATTUS00541]	104	701
[RATTUS00547]	98	865
[RATTUS00553]	210	738
[RATTUS00557]	48	191
[RATTUS00562]	58	471
[RATTUS00575]	17	145
[RATTUS00577]	4	23
[RATTUS00578]	138	8813

FIG. 16AM

[RATTUS00581]	288	1354
[RATTUS00587]	29	106
[RATTUS00598]	51	492
[RATTUS00606]	57	1463
[RATTUS00607]	-1	27
[RATTUS00608]	255	1513
[RATTUS00618]	135	666
[RATTUS00627]	16	47
[RATTUS00628]	7803	65472
[RATTUS00629]	18	62
[RATTUS00633]	108	400
[RATTUS00643]	7	54
[RATTUS00645]	91	818
[RATTUS00648]	32	218
[RATTUS00656]	331	1437
[RATTUS00664]	90	310
[RATTUS00691]	5	43
[RATTUS00696]	50	152
[RATTUS00704]	207	1531
[RATTUS00717]	295	3005
[RATTUS00724]	-2	60
[RATTUS00728]	382	3503
[RATTUS00729]	57	170
[RATTUS00733]	2	28
[RATTUS00735]	35	422
[RATTUS00737]	149	798
[RATTUS00739]	10	210
[RATTUS00742]	49	177
[RATTUS00743]	917	11068
[RATTUS00744]	20	58
[RATTUS00746]	11	48
[RATTUS00747]	89	1285
[RATTUS00753]	45	147
[RATTUS00755]	37	131
[RATTUS00758]	88	3600
[RATTUS00761]	38	118
[RATTUS00762]	211	911
[RATTUS00768]	10	28
[RATTUS00769]	14	69
[RATTUS00770]	73	317
[RATTUS00773]	66	624
[RATTUS00778]	1	8
[RATTUS00779]	249	825
[RATTUS00780]	1	8
[RATTUS00781]	180	688
[RATTUS00784]	52	358
[RATTUS00788]	6	32
[RATTUS00794]	600	1903
[RATTUS00795]	5	33
[RATTUS00801]	357	1842
[RATTUS00812]	-2	15
[RATTUS00816]	-1	14

FIG. 16AN

[RATTUS00817]	8	118
[RATTUS00819]	2	63
[RATTUS00828]	10	71
[RATTUS00829]	31	121
[RATTUS00831]	559	1661
[RATTUS00832]	2	68
[RATTUS00840]	186	866
[RATTUS00843]	46	205
[RATTUS00848]	3319	21622
[RATTUS00855]	1724	7891
[RATTUS00859]	47	195
[RATTUS00862]	3	74
[RATTUS00867]	180	994
[RATTUS00868]	46	134
[RATTUS00870]	1095	6192
[RATTUS00871]	6	48
[RATTUS00872]	20	83
[RATTUS00873]	1108	4980
[RATTUS00875]	7	58
[RATTUS00884]	23	130
[RATTUS00889]	-5	36
[RATTUS00893]	210	2819
[RATTUS00902]	606	2446
[RATTUS00905]	363	1883
[RATTUS00913]	184	813
[RATTUS00914]	11	105
[RATTUS00917]	52	259
[RATTUS00918]	47	270
[RATTUS00919]	133	1200
[RATTUS00921]	16	73
[RATTUS00923]	639	3731
[RATTUS00926]	158	807
[RATTUS00940]	6	169
[RATTUS00946]	32	247
[RATTUS00970]	5	107
[RATTUS00971]	62	272
[RATTUS00974]	30	294
[RATTUS00975]	313	1590
[RATTUS00976]	139	667
[RATTUS00979]	10	73
[RATTUS00982]	74	565
[RATTUS00983]	-1	22
[RATTUS00986]	45	145
[RATTUS00991]	533	1769
[RATTUS00992]	68	447
[RATTUS01007]	15	69
[RATTUS01009]	22	75
[RATTUS01024]	318	1080
[RATTUS01032]	45	372
[RATTUS01040]	35	105
[RATTUS01041]	145	890
[RATTUS01052]	585	7213

FIG. 16AO

[RATTUS01056]	99	403
[RATTUS01057]	11	225
[RATTUS01058]	26	184
[RATTUS01062]	15	91
[RATTUS01067]	102	1353
[RATTUS01069]	312	1560
[RATTUS01070]	17	64
[RATTUS01071]	1152	48552
[RATTUS01077]	26	102
[RATTUS01086]	3	22
[RATTUS01087]	329	961
[RATTUS01088]	260	828
[RATTUS01093]	152	521
[RATTUS01096]	238	748
[RATTUS01099]	5	24
[RATTUS01103]	8	53
[RATTUS01104]	302	2714
[RATTUS01105]	54	375
[RATTUS01106]	22	70
[RATTUS01112]	8	42
[RATTUS01113]	0	23
[RATTUS01122]	36	181
[RATTUS01123]	11	91
[RATTUS01133]	1260	10710
[RATTUS01138]	502	2271
[RATTUS01139]	123	460
[RATTUS01144]	110	885
[RATTUS01146]	16	83
[RATTUS01155]	145	719
[RATTUS01167]	37	162
[RATTUS01170]	397	5996
[RATTUS01175]	256	1010
[RATTUS01176]	13	69
[RATTUS01178]	267	1057
[RATTUS01179]	13	39
[RATTUS01180]	203	2899
[RATTUS01182]	7	24
[RATTUS01183]	39	150
[RATTUS01185]	423	1775
[RATTUS01186]	155	904
[RATTUS01187]	739	9351
[RATTUS01204]	77	383
[RATTUS01205]	389	1145
[RATTUS01210]	78	234
[RATTUS01211]	39	146
[RATTUS01219]	569	2241
[RATTUS01220]	196	2533
[RATTUS01221]	1180	14171
[RATTUS01225]	16	88
[RATTUS01230]	39	210
[RATTUS01233]	45	332
[RATTUS01247]	39	224

FIG. 16AP

[RATTUS01248]	53	213
[RATTUS01251]	-8	102
[RATTUS01255]	6	20
[RATTUS01267]	601	2038
[RATTUS01275]	282	1076
[RATTUS01281]	31	157
[RATTUS01282]	17	73
[RATTUS01283]	4	82
[RATTUS01289]	6	55
[RATTUS01292]	51	165
[RATTUS01294]	123	436
[RATTUS01295]	53	196
[RATTUS01296]	2470	62592
[RATTUS01297]	189	1130
[RATTUS01298]	55	376
[RATTUS01303]	413	3968
[RATTUS01306]	108	760
[RATTUS01312]	8	104
[RATTUS01315]	58	225
[RATTUS01316]	10	31
[RATTUS01318]	14	80
[RATTUS01319]	1160	5182
[RATTUS01326]	4	25
[RATTUS01336]	0	22
[RATTUS01349]	599	12214
[RATTUS01356]	22	125
[RATTUS01358]	4773	31038
[RATTUS01363]	320	1523
[RATTUS01367]	26	184
[RATTUS01373]	37	108
[RATTUS01384]	52	743
[RATTUS01388]	2	13
[RATTUS01395]	4	175
[RATTUS01397]	16	67
[RATTUS01400]	229	1390
[RATTUS01402]	47	198
[RATTUS01404]	468	2031
[RATTUS01412]	71	293
[RATTUS01413]	32	102
[RATTUS01414]	51	2700
[RATTUS01416]	217	632
[RATTUS01417]	144	737
[RATTUS01418]	329	1372
[RATTUS01422]	78	321
[RATTUS01426]	21	247
[RATTUS01429]	46	190
[RATTUS01440]	108	426
[RATTUS01442]	3	124
[RATTUS01444]	52	189
[RATTUS01446]	444	1781
[RATTUS01447]	272	2321
[RATTUS01451]	122	579

FIG. 16AQ

[RATTUS01454]	108	533
[RATTUS01455]	32	240
[RATTUS01456]	38	308
[RATTUS01457]	264	903
[RATTUS01461]	308	1886
[RATTUS01462]	6	45
[RATTUS01467]	450	3068
[RATTUS01469]	259	1277
[RATTUS01471]	24	72
[RATTUS01475]	34	156
[RATTUS01478]	6	61
[RATTUS01489]	461	2039
[RATTUS01490]	30	182
[RATTUS01493]	80	710
[RATTUS01495]	6	67
[RATTUS01501]	2	23
[RATTUS01502]	35	151
[RATTUS01503]	56	292
[RATTUS01506]	42	130
[RATTUS01508]	14	58
[RATTUS01509]	50	225
[RATTUS01511]	115	581
[RATTUS01513]	29	89
[RATTUS01516]	117	450
[RATTUS01517]	33	150
[RATTUS01519]	17	2240
[RATTUS01520]	270	1161
[RATTUS01526]	457	13498
[RATTUS01529]	39	131
[RATTUS01530]	61	645
[RATTUS01533]	15	59
[RATTUS01535]	6	39
[RATTUS01538]	115	2123
[RATTUS01539]	944	2970
[RATTUS01541]	120	625
[RATTUS01545]	11	67
[RATTUS01549]	7	55
[RATTUS01555]	471	1841
[RATTUS01557]	18	153
[RATTUS01558]	272	1489
[RATTUS01561]	8	122
[RATTUS01564]	66	283
[RATTUS01565]	9	112
[RATTUS01572]	110	448
[RATTUS01579]	98	988
[RATTUS01580]	21	120
[RATTUS01590]	38	138
[RATTUS01592]	42	321
[RATTUS01593]	7	27
[RATTUS01595]	19	2185
[RATTUS01596]	75	323
[RATTUS01597]	30	271

FIG. 16AR

[RATTUS01601]	16	47
[RATTUS01602]	11	109
[RATTUS01603]	843	3971
[RATTUS01604]	71	258
[RATTUS01611]	29	119
[RATTUS01615]	1444	5701
[RATTUS01619]	20175	65460
[RATTUS01621]	9	78
[RATTUS01622]	273	870
[RATTUS01626]	38	145
[RATTUS01627]	0	8
[RATTUS01629]	528	1742
[RATTUS01631]	1317	8652
[RATTUS01632]	127	742
[RATTUS01633]	285	1848
[RATTUS01638]	32	527
[RATTUS01641]	18	127
[RATTUS01645]	25	83
[RATTUS01650]	240	5799
[RATTUS01663]	8	27
[RATTUS01664]	66	2138
[RATTUS01666]	18	92
[RATTUS01669]	1498	14186
[RATTUS01672]	96	569
[RATTUS01680]	91	406
[RATTUS01683]	52	262
[RATTUS01690]	21	66
[RATTUS01692]	105	446
[RATTUS01693]	0	9
[RATTUS01694]	2	28
[RATTUS01700]	73	1518
[RATTUS01707]	5	36
[RATTUS01708]	17	55
[RATTUS01709]	499	1839
[RATTUS01713]	3181	38974
[RATTUS01714]	489	4594
[RATTUS01720]	823	2516
[RATTUS01724]	48	162
[RATTUS01726]	8	30
[RATTUS01730]	9	40
[RATTUS01731]	2	38
[RATTUS01734]	95	1348
[RATTUS01736]	192	1659
[RATTUS01737]	143	766
[RATTUS01739]	17	101
[RATTUS01740]	2	14
[RATTUS01743]	14	66
[RATTUS01747]	121	562
[RATTUS01750]	415	2415
[RATTUS01751]	91	704
[RATTUS01756]	32	111
[RATTUS01758]	1795	6234

FIG. 16AS

[RATTUS01759]	15	101
[RATTUS01762]	949	3250
[RATTUS01765]	433	1319
[RATTUS01768]	7	25
[RATTUS01774]	37	116
[RATTUS01779]	2558	27047
[RATTUS01781]	6	19
[RATTUS01782]	47	194
[RATTUS01786]	9	30
[RATTUS01797]	-9	193
[RATTUS01799]	197	1281
[RATTUS01806]	13	104
[RATTUS01811]	159	1015
[RATTUS01816]	6	33
[RATTUS01818]	23	75
[RATTUS01820]	44	316
[RATTUS01823]	38	148
[RATTUS01825]	46	174
[RATTUS01827]	2	68
[RATTUS01829]	9	34
[RATTUS01831]	15	98
[RATTUS01833]	47	504
[RATTUS01834]	312	1693
[RATTUS01837]	46	267
[RATTUS01839]	37	298
[RATTUS01845]	251	2468
[RATTUS01846]	1554	6957
[RATTUS01849]	50	282
[RATTUS01856]	76	368
[RATTUS01861]	128	559
[RATTUS01862]	32	208
[RATTUS01863]	8	25
[RATTUS01870]	596	4661
[RATTUS01873]	11	109
[RATTUS01875]	27	85
[RATTUS01878]	62	215
[RATTUS01879]	1559	7939
[RATTUS01881]	156	821
[RATTUS01882]	220	646
[RATTUS01884]	231	1597
[RATTUS01885]	1528	7771
[RATTUS01892]	1	28
[RATTUS01895]	69	238
[RATTUS01902]	490	3302
[RATTUS01915]	9	36
[RATTUS01917]	200	597
[RATTUS01929]	75	338
[RATTUS01930]	172	1274
[RATTUS01932]	572	1680
[RATTUS01933]	19	158
[RATTUS01935]	560	2459
[RATTUS01940]	305	1112

FIG. 16AT

[RATTUS01944]	270	4222
[RATTUS01946]	9	55
[RATTUS01951]	103	591
[RATTUS01955]	13	42
[RATTUS01970]	30	102
[RATTUS01974]	31	229
[RATTUS01977]	40	123
[RATTUS01978]	7	35
[RATTUS01980]	7	33
[RATTUS01981]	14	47
[RATTUS01993]	8	41
[RATTUS01996]	9	125
[RATTUS02006]	93	982
[RATTUS02009]	233	1485
[RATTUS02012]	47	391
[RATTUS02013]	532	2046
[RATTUS02017]	94	435
[RATTUS02018]	55	191
[RATTUS02019]	47	752
[RATTUS02020]	1204	3725
[RATTUS02024]	4	106
[RATTUS02027]	590	2118
[RATTUS02028]	26	118
[RATTUS02031]	217	1046
[RATTUS02032]	42	296
[RATTUS02034]	-1	35
[RATTUS02038]	215	1194
[RATTUS02040]	7	41
[RATTUS02044]	6	34
[RATTUS02049]	5	23
[RATTUS02051]	456	2241
[RATTUS02056]	14892	43075
[RATTUS02067]	21	124
[RATTUS02072]	469	2732
[RATTUS02074]	77	301
[RATTUS02075]	0	10
[RATTUS02076]	231	1062
[RATTUS02078]	13	47
[RATTUS02079]	43	279
[RATTUS02081]	2	11
[RATTUS02083]	44	128
[RATTUS02085]	4	64
[RATTUS02091]	258	1135
[RATTUS02094]	269	790
[RATTUS02101]	-1	18
[RATTUS02102]	137	495
[RATTUS02103]	11	43
[RATTUS02104]	8	25
[RATTUS02105]	37	123
[RATTUS02106]	16	70
[RATTUS02107]	28	205
[RATTUS02109]	-4	44

FIG. 16AU

[RATTUS02122]	44	975
[RATTUS02123]	6	52
[RATTUS02124]	589	3277
[RATTUS02125]	389	2128
[RATTUS02126]	16	128
[RATTUS02128]	296	1837
[RATTUS02129]	90	325
[RATTUS02130]	596	2061
[RATTUS02131]	57	373
[RATTUS02132]	12	93
[RATTUS02136]	249	2389
[RATTUS02139]	31	96
[RATTUS02141]	25	97
[RATTUS02143]	304	1049
[RATTUS02145]	25	91
[RATTUS02156]	72	395
[RATTUS02157]	150	1237
[RATTUS02160]	26	78
[RATTUS02164]	150	1199
[RATTUS02167]	41	177
[RATTUS02168]	2736	25516
[RATTUS02169]	3	35
[RATTUS02170]	77	263
[RATTUS02180]	194	619
[RATTUS02183]	764	6562
[RATTUS02185]	7	65
[RATTUS02188]	307	1153
[RATTUS02192]	-1	43
[RATTUS02194]	207	1508
[RATTUS02195]	13	79
[RATTUS02202]	7	52
[RATTUS02208]	8	27
[RATTUS02209]	6	23
[RATTUS02214]	12	120
[RATTUS02217]	21	68
[RATTUS02224]	10	84
[RATTUS02226]	192	904
[RATTUS02227]	20	58
[RATTUS02228]	495	1809
[RATTUS02229]	16	86
[RATTUS02230]	49	173
[RATTUS02231]	149	480
[RATTUS02233]	22558	65462
[RATTUS02234]	4	28
[RATTUS02235]	258	4984
[RATTUS02236]	57	332
[RATTUS02238]	3	52
[RATTUS02260]	22	129
[RATTUS02269]	146	682
[RATTUS02279]	75	317
[RATTUS02292]	51	258
[RATTUS02293]	-4	16

FIG. 16AV

[RATTUS02300]	34	175
[RATTUS02302]	72	667
[RATTUS02316]	-3	17
[RATTUS02318]	11	34
[RATTUS02321]	6	37
[RATTUS02329]	-2	20
[RATTUS02330]	171	502
[RATTUS02332]	5	152
[RATTUS02333]	2084	6856
[RATTUS02335]	4	21
[RATTUS02336]	9	53
[RATTUS02339]	6	26
[RATTUS02341]	-109	31
[RATTUS02342]	8	54
[RATTUS02344]	1	22
[RATTUS02345]	10918	43503
[RATTUS02349]	257	1034
[RATTUS02360]	61	322
[RATTUS02366]	42	319
[RATTUS02378]	25	117
[RATTUS02383]	954	6243
[RATTUS02393]	30	112
[RATTUS02397]	2	26
[RATTUS02400]	33	123
[RATTUS02405]	28	148
[RATTUS02407]	5	16
[RATTUS02408]	24	159
[RATTUS02409]	10	92
[RATTUS02411]	10	57
[RATTUS02412]	3	30
[RATTUS02413]	11	55
[RATTUS02416]	3145	53458
[RATTUS02418]	90	532
[RATTUS02424]	25	97
[RATTUS02428]	135	586
[RATTUS02440]	3103	17343
[RATTUS02444]	879	2907
[RATTUS02445]	1234	6563
[RATTUS02447]	1637	10947
[RATTUS02448]	26	87
[RATTUS02450]	1	42
[RATTUS02455]	56	178
[RATTUS02458]	-2	-7
[RATTUS02461]	9	51
[RATTUS02462]	59	364
[RATTUS02467]	62	209
[RATTUS02478]	9	98
[RATTUS02481]	234	5847
[RATTUS02482]	201	1179
[RATTUS02490]	267	2903
[RATTUS02493]	187	1190
[RATTUS02499]	55	216

FIG. 16AW

[RATTUS02503]	185	642
[RATTUS02511]	10	66
[RATTUS02518]	-1	8
[RATTUS02525]	127	382
[RATTUS02533]	10	29
[RATTUS02539]	521	1763
[RATTUS02542]	60	645
[RATTUS02554]	36	121
[RATTUS02556]	57	304
[RATTUS02560]	8	74
[RATTUS02562]	82	363
[RATTUS02566]	63	410
[RATTUS02569]	225	791
[RATTUS02571]	29	103
[RATTUS02572]	4	63
[RATTUS02578]	19	171
[RATTUS02583]	258	1639
[RATTUS02584]	3	11
[RATTUS02587]	6	25
[RATTUS02598]	10	55
[RATTUS02602]	-1	13
[RATTUS02603]	97	1320
[RATTUS02604]	61	209
[RATTUS02606]	241	1867
[RATTUS02612]	25	186
[RATTUS02648]	34	126
[RATTUS02653]	543	3633
[RATTUS02655]	538	1871
[RATTUS02660]	141	526
[RATTUS02661]	15	119
[RATTUS02662]	131	596
[RATTUS02667]	84	361
[RATTUS02668]	80	474
[RATTUS02669]	33	124
[RATTUS02670]	8	48
[RATTUS02675]	303	1032
[RATTUS02689]	438	1887
[RATTUS02693]	7	27
[RATTUS02695]	37	113
[RATTUS02697]	6	54
[RATTUS02698]	1	25
[RATTUS02702]	43	152
[RATTUS02704]	45	224
[RATTUS02712]	188	8103
[RATTUS02717]	67	1768
[RATTUS02718]	11	254
[RATTUS02725]	18	69
[RATTUS02730]	71	293
[RATTUS02734]	15	59
[RATTUS02740]	27	237
[RATTUS02745]	8	58
[RATTUS02749]	25	145

FIG. 16AX

[RATTUS02764]	33	98
[RATTUS02766]	5532	41626
[RATTUS02779]	22	146
[RATTUS02785]	65	729
[RATTUS02797]	168	1893
[RATTUS02812]	331	1104
[RATTUS02822]	8	38
[RATTUS02825]	11	57
[RATTUS02828]	1	25
[RATTUS02861]	137	634
[RATTUS02865]	530	2364
[RATTUS02872]	124	1784
[RATTUS02877]	117	615
[RATTUS02879]	134	842
[RATTUS02880]	2	47
[RATTUS02882]	168	531
[RATTUS02883]	34	217
[RATTUS02895]	59	278
[RATTUS02904]	2	25
[RATTUS02905]	701	2579
[RATTUS02907]	34	112
[RATTUS02911]	649	3202
[RATTUS02914]	254	1814
[RATTUS02915]	7	128
[RATTUS02918]	298	1992
[RATTUS02921]	311	1321
[RATTUS02924]	215	1225
[RATTUS02926]	221	1289
[RATTUS02931]	154	750
[RATTUS02933]	11	76
[RATTUS02940]	9	30
[RATTUS02943]	641	4037
[RATTUS02949]	99	729
[RATTUS02960]	129	625
[RATTUS02962]	111	583
[RATTUS02964]	4	60
[RATTUS02972]	41	131
[RATTUS02981]	4	56
[RATTUS02982]	36	251
[RATTUS02983]	17	214
[RATTUS02986]	59	174
[RATTUS02987]	16	61
[RATTUS02999]	251	818
[RATTUS03019]	366	1332
[RATTUS03020]	154	708
[RATTUS03021]	123	486
[RATTUS03024]	-4	9
[RATTUS03025]	113	482
[RATTUS03033]	25	127
[RATTUS03037]	111	915
[RATTUS03040]	86	332
[RATTUS03041]	5	46

FIG. 16AY

[RATTUS03046]	147	649
[RATTUS03048]	78	482
[RATTUS03052]	945	3121
[RATTUS03058]	29	95
[RATTUS03069]	3	12
[RATTUS03073]	5	39
[RATTUS03075]	0	32
[RATTUS03079]	134	776
[RATTUS03083]	-4	13
[RATTUS03094]	2	39
[RATTUS03095]	3790	29872
[RATTUS03099]	25	112
[RATTUS03107]	43	125
[RATTUS03109]	12	59
[RATTUS03110]	455	1530
[RATTUS03124]	116	393
[RATTUS03128]	45	149
[RATTUS03137]	124	978
[RATTUS03148]	24	88
[RATTUS03150]	83	481
[RATTUS03158]	75	303
[RATTUS03163]	199	628
[RATTUS03175]	973	5716
[RATTUS03179]	250	1482
[RATTUS03185]	6708	46991
[RATTUS03192]	5	22
[RATTUS03198]	-10	27
[RATTUS03199]	720	6237
[RATTUS03202]	6	33
[RATTUS03203]	561	1752
[RATTUS03208]	395	2356
[RATTUS03210]	3	16
[RATTUS03214]	909	7061
[RATTUS03220]	35	171
[RATTUS03223]	54	233
[RATTUS03230]	117	373
[RATTUS03238]	0	12
[RATTUS03239]	1735	8301
[RATTUS03241]	104	310
[RATTUS03250]	168	532
[RATTUS03255]	30	314
[RATTUS03256]	15	44
[RATTUS03265]	14	83
[RATTUS03267]	47	179
[RATTUS03270]	366	2035
[RATTUS03272]	4	35
[RATTUS03274]	16	87
[RATTUS03275]	1	24
[RATTUS03276]	30	101
[RATTUS03279]	163	1357
[RATTUS03280]	54	321
[RATTUS03282]	54	426

FIG. 16AZ

[RATTUS03283]	43	367
[RATTUS03284]	66	212
[RATTUS03288]	7	162
[RATTUS03290]	0	4
[RATTUS03300]	5	56
[RATTUS03309]	55	170
[RATTUS03310]	-1	47
[RATTUS03315]	103	690
[RATTUS03319]	66	275
[RATTUS03322]	167	812
[RATTUS03324]	19	221
[RATTUS03326]	160	3246
[RATTUS03327]	617	1976
[RATTUS03333]	4079	65330
[RATTUS03341]	420	3783
[RATTUS03346]	31	100
[RATTUS03348]	119	731
[RATTUS03355]	16	64
[RATTUS03357]	215	835
[RATTUS03366]	12	55
[RATTUS03378]	12	87
[RATTUS03382]	88	433
[RATTUS03391]	57	186
[RATTUS03396]	130	468
[RATTUS03398]	3	52
[RATTUS03399]	1922	24122
[RATTUS03400]	10	37
[RATTUS03404]	2	30
[RATTUS03408]	7	21
[RATTUS03410]	367	1074
[RATTUS03415]	-1	84
[RATTUS03416]	35	138
[RATTUS03434]	-3	14
[RATTUS03443]	14	50
[RATTUS03446]	147	1175
[RATTUS03452]	164	883
[RATTUS03457]	7	43
[RATTUS03472]	212	1472
[RATTUS03479]	6	108
[RATTUS03494]	17	128
[RATTUS03495]	45	458
[RATTUS03501]	279	2405
[RATTUS03504]	3	13
[RATTUS03506]	3473	22582
[RATTUS03510]	1650	32050
[RATTUS03515]	28	95
[RATTUS03516]	49	258
[RATTUS03522]	240	743
[RATTUS03524]	9	59
[RATTUS03541]	25	261
[RATTUS03549]	78	2876
[RATTUS03553]	21	131

FIG. 16AAA

[RATTUS03558]	3	59
[RATTUS03560]	106	353
[RATTUS03561]	231	3141
[RATTUS03568]	23	117
[RATTUS03575]	574	2521
[RATTUS03585]	38	334
[RATTUS03588]	297	2452
[RATTUS03589]	3	17
[RATTUS03594]	143	498
[RATTUS03595]	54	155
[RATTUS03596]	78	558
[RATTUS03606]	7	62
[RATTUS03608]	76	521
[RATTUS03612]	12	60
[RATTUS03614]	13	40
[RATTUS03615]	8	31
[RATTUS03616]	5	35
[RATTUS03620]	316	4539
[RATTUS03624]	82	343
[RATTUS03625]	10	95
[RATTUS03631]	22	142
[RATTUS03633]	153	1949
[RATTUS03634]	5518	63943
[RATTUS03643]	39	123
[RATTUS03647]	-1	38
[RATTUS03649]	0	24
[RATTUS03651]	-3	39
[RATTUS03653]	92	584
[RATTUS03660]	32	103
[RATTUS03667]	25	118
[RATTUS03669]	233	1455
[RATTUS03670]	67	304
[RATTUS03671]	86	413
[RATTUS03672]	30	87
[RATTUS03674]	52	211
[RATTUS03675]	600	2273
[RATTUS03676]	21	232
[RATTUS03678]	45	295
[RATTUS03681]	2	17
[RATTUS03684]	4	24
[RATTUS03693]	226	1219
[RATTUS03697]	11	90
[RATTUS03705]	98	681
[RATTUS03706]	17	146
[RATTUS03710]	15	63
[RATTUS03717]	26	225
[RATTUS03718]	0	69
[RATTUS03724]	165	1000
[RATTUS03725]	88	388
[RATTUS03727]	24	114
[RATTUS03733]	418	2734
[RATTUS03735]	3935	16175

FIG. 16AAB

[RATTUS03739]	39	131
[RATTUS03741]	3535	11437
[RATTUS03749]	16	62
[RATTUS03772]	92	468
[RATTUS03783]	11	53
[RATTUS03785]	508	1691
[RATTUS03790]	215	1157
[RATTUS03794]	44	129
[RATTUS03808]	5	149
[RATTUS03812]	15	180
[RATTUS03813]	83	590
[RATTUS03816]	28	203
[RATTUS03819]	50	283
[RATTUS03823]	10	54
[RATTUS03828]	36	173
[RATTUS03830]	2750	12902
[RATTUS03834]	178	1464
[RATTUS03836]	127	2057
[RATTUS03844]	28	101
[RATTUS03848]	28	129
[RATTUS03849]	136	539
[RATTUS03852]	84	543
[RATTUS03860]	63	2497
[RATTUS03866]	1169	4434
[RATTUS03901]	84	539
[RATTUS03902]	20	353
[RATTUS03910]	17	54
[RATTUS03914]	5	21
[RATTUS03917]	81	275
[RATTUS03932]	60	227
[RATTUS03950]	572	2257
[RATTUS03951]	257	1457
[RATTUS03967]	5	37
[RATTUS03971]	272	3583
[RATTUS03972]	3	84
[RATTUS03976]	9	67
[RATTUS03982]	142	683
[RATTUS03992]	552	1706
[RATTUS03999]	25	127
[RATTUS04003]	7	25
[RATTUS04013]	46	177
[RATTUS04015]	1792	6376
[RATTUS04017]	13	72
[RATTUS04018]	-7	63
[RATTUS04020]	444	2229
[RATTUS04025]	2047	30169
[RATTUS04028]	61	238
[RATTUS04029]	82	396
[RATTUS04032]	4187	18659
[RATTUS04033]	60	272
[RATTUS04034]	5	60
[RATTUS04041]	338	2518

FIG. 16AAC

[RATTUS04050]	173	712
[RATTUS04052]	542	4475
[RATTUS04067]	8	50
[RATTUS04069]	4	27
[RATTUS04076]	53	205
[RATTUS04082]	154	531
[RATTUS04084]	2	9
[RATTUS04097]	215	753
[RATTUS04100]	12	49
[RATTUS04103]	39	141
[RATTUS04104]	4	67
[RATTUS04119]	5272	22084
[RATTUS04120]	152	941
[RATTUS04122]	3	160
[RATTUS04134]	23	131
[RATTUS04138]	62	191
[RATTUS04142]	4	17
[RATTUS04148]	60	216
[RATTUS04149]	6	56
[RATTUS04151]	29	142
[RATTUS04154]	7	46
[RATTUS04155]	56	171
[RATTUS04158]	49	309
[RATTUS04162]	205	1176
[RATTUS04163]	114	875
[RATTUS04165]	203	757
[RATTUS04166]	210	1595
[RATTUS04171]	38	941
[RATTUS04173]	33	110
[RATTUS04176]	11	87
[RATTUS04177]	34	165
[RATTUS04180]	17	64
[RATTUS04181]	48	168
[RATTUS04183]	73	285
[RATTUS04184]	3	14
[S40716]	6	58
[S44194]	147	517
[S45812]	10	61
[S48142]	322	1118
[S49760]	312	937
[S50879]	488	2958
[S57790]	54	320
[S63458]	239	1136
[S63519]	8	23
[S67620]	31	133
[S68589]	0	26
[S68987]	-1	22
[S70355]	211	928
[S71275]	24	194
[S71286]	8	33
[S71317]	185	1116
[S71523]	2	97

FIG. 16AAD

[S71570]	11	65
[S73608]	624	3356
[S73713]	6	22
[S73971]	8	41
[S74029]	40	180
[S74907]	36	125
[S75275]	33	113
[S75437]	17	106
[S75997]	1486	6051
[S76214]	50	160
[S76742]	1	28
[S76779]	50	185
[S77793]	6476	43872
[S78744]	18	124
[S79053]	270	1496
[S79214]	-1	26
[S79307]	8304	44423
[S79711]	80	591
[S79794]	50	190
[S79820]	1397	6146
[S80118]	167	948
[S80439]	29	133
[S81002]	11	43
[S81842]	57	294
[S82911]	1	35
[S83320]	159	715
[S98336]	173	1305
[U00620]	69	304
[U01280]	540	1772
[U02316]	8	63
[U02534]	4743	65466
[U03628]	1425	5132
[U03629]	118	858
[U04317]	923	4816
[U04933]	7	72
[U04980]	68	248
[U06103]	148	844
[U06434]	44	374
[U08031]	150	2492
[U08214]	8	194
[U08263]	132	1600
[U09021]	64	476
[U12571]	12	68
[U15408]	232	1577
[U15425]	145	1310
[U17013]	13	81
[U17375]	39	210
[U18293]	85	464
[U18650]	104	352
[U19614]	147	602
[U20121]	5	56
[U20286]	52	352

FIG. 16AAE

[U20298]	61	268
[U21116]	61	237
[U21954]	29	158
[U22830]	161	1264
[U23377]	108	456
[U23407]	163	624
[U23438]	166	635
[U24489]	763	2674
[U25148]	1023	2971
[U25281]	83	651
[U25282]	67	302
[U27186]	61	351
[U29174]	87	357
[U30831]	8	47
[U31168]	5	21
[U31815]	1	25
[U31816]	197	962
[U32434]	17	65
[U32436]	226	712
[U35174]	7	65
[U36897]	-1	14
[U37540]	3	48
[U38376]	21	71
[U39206]	100	385
[U39208]	36	346
[U39555]	5	47
[U39571]	419	1320
[U39572]	133	1743
[U39608]	-3	38
[U40627]	242	981
[U41183]	781	2653
[U41663]	174	1222
[U41803]	209	2535
[U41853]	1	50
[U42581]	20	264
[U45479]	4	22
[U45986]	7265	36943
[U47331]	2993	42020
[U47672]	-400	1374
[U48809]	340	1654
[U50842]	14	72
[U51013]	47	273
[U51017]	18	91
[U51583]	12	38
[U52218]	36	123
[U53513]	7305	65463
[U53822]	185	1439
[U53873]	21	121
[U53927]	59	280
[U55765]	41	181
[U55816]	1393	4645
[U55849]	169	1267

FIG. 16AAF

[U55995]	454	3386
[U56261]	25	121
[U56732]	19	312
[U56822]	182	1255
[U56824]	943	14128
[U56859]	305	1197
[U56936]	67	2641
[U57049]	40	474
[U57062]	260	1356
[U57063]	5	89
[U57391]	31	110
[U59456]	32	111
[U60562]	4	41
[U60564]	-4	26
[U61157]	10	37
[U61261]	1048	6614
[U61743]	2994	27499
[U62315]	64	320
[U62316]	4	16
[U63839]	5	20
[U65417]	725	3823
[U66470]	6	30
[U66566]	94	1385
[U66567]	21	86
[U67084]	43	382
[U67140]	74	700
[U67863]	5	20
[U67914]	114	686
[U67915]	304	899
[U67995]	10	69
[U68490]	668	2736
[U68544]	176	529
[U68726]	176	942
[U72632]	2	10
[U72741]	2	62
[U73503]	2	66
[U74586]	2	11
[U75899]	6	135
[U75923]	54	177
[U76557]	64	301
[U76997]	31	312
[U77583]	22	103
[U77697]	3	42
[U77699]	8	62
[U77931]	11	34
[U78090]	848	7564
[U78113]	116	645
[U78116]	372	3007
[U78117]	48	295
[U78118]	218	3059
[U78121]	51	609
[U78123]	426	14820

FIG. 16AAG

[U78125]	555	3930
[U78126]	23	213
[U78127]	5	43
[U78129]	206	933
[U78130]	53	1231
[U78134]	52	277
[U78136]	18	60
[U78144]	606	4123
[U78145]	5	96
[U78146]	210	1493
[U78304]	39	343
[U81036]	10	64
[U81037]	128	868
[U83139]	8	47
[U83666]	94	459
[U85512]	42	244
[U85606]	7	26
[U85959]	12	64
[U87598]	29	123
[U88622]	29	422
[U89280]	12	87
[U89743]	38	125
[U89744]	389	1343
[U89745]	210	839
[U90444]	14	264
[U91679]	77	368
[U92010]	3412	12452
[U92802]	36	455
[U94856]	5542	40104
[U94858]	8106	41670
[U95147]	17.5 (	63
[U96920]	136	843
[U97061]	296	1420
[V01237]	90	669
[V01255]	6	62
[V01256]	1065	33594
[X00306]	128	1820
[X01958]	28	166
[X02284]	86	496
[X02341]	392	1991
[X02411]	39	208
[X03032]	101	883
[X06108]	819	4722
[X06769]	32	270
[X07314]	53	256
[X13016]	697	6598
[X13119]	378	2346
[X13295]	25	76
[X13905]	33	104
[X14182]	-6	19
[X14211]	827	4567
[X14318]	530	4576

FIG. 16AAH

[X16261]	344	2092
[X52757]	58	193
[X52772]	14	100
[X52815]	392	1644
[X53052]	150	784
[X53054]	1177	31149
[X53087]	30	87
[X53261]	49	279
[X53949]	1158	5792
[X54250]	24	91
[X54467]	89	403
[X55180]	26	86
[X56133]	-4	5
[X56159]	26	79
[X56326]	51	335
[X56448]	20	85
[X56747]	3	21
[X57405]	41	278
[X59012]	393	1761
[X59014]	3	10
[X59290]	254	1664
[X59864]	223	3172
[X59993]	130	741
[X60658]	64	203
[X60659]	159	928
[X60660]	25	125
[X61106]	11	120
[X61381]	40	172
[X61479]	49	291
[X62327]	16	49
[X62404]	60	201
[X63911]	81	608
[X65036]	23	104
[X68101]	22	113
[X68394]	12	37
[X68782]	42	200
[X69834]	3	14
[X70369]	42	310
[X70706]	9	71
[X71442]	63	896
[X73292]	55	192
[X74229]	77	1026
[X74293]	149	1370
[X74294]	43	279
[X74815]	1064	17888
[X76129]	60	319
[X76489]	-2	62
[X77236]	121	546
[X77815]	5262	35820
[X78595]	4	26
[X78985]	19	82
[X79881]	6	31

FIG. 16AAI

[X79991]	1	47
[X80029]	349	1425
[X81301]	658	6049
[X81316]	7	29
[X81825]	414	1821
[X82152]	97	498
[X82777]	24	76
[X83546]	89	296
[X83671]	86	373
[X84210]	149	619
[X89638]	24	127
[X89694]	149	836
[X89695]	93	678
[X89698]	26	327
[X89702]	78	251
[X89705]	3635	12149
[X89706]	68	382
[X89962]	38	344
[X90475]	77	612
[X90845]	16	51
[X94186]	23	74
[X94769]	4	78
[X96790]	14	63
[X98234]	278	2640
[X98746]	280	873
[X99901]	7	47
[Y07783]	34	114
[Y07832]	5	148
[Y08882]	159	2661
[Y08883]	2434	17907
[Y08981]	53	315
[Y09049]	36	226
[Y09111]	7	34
[Y09175]	125	926
[Y09176]	3	19
[Y09177]	62	590
[Y09945]	130	861
[Y11490]	103	299
[Y11491]	3945	12827
[Y14635]	1620	4968
[Y15054]	23	141
[Y17048]	5	51
[Y17258]	89	547
[Y17326]	-1	36
[Y17327]	29	190
[Y18208]	55	655
[Y18571]	43	395
[Y18572]	35	137
[Z11504]	43	332
[Z11994]	18	185
[Z12019]	8	46
[Z12298]	18	75

FIG. 16AAJ

[Z14117]	45	152
[Z14119]	7	34
[Z15029]	177	981
[Z15158]	1542	19835
[Z23272]	514	1676
[Z29072]	571	14449
[Z30584]	156	1040
[Z35139]	855	9627

FIG. 16AAK

Ion Channels in PTB Cluster

proton gated cation channel	Novel
Sodium channel, nonvoltage-gated	Novel
calcium channel, voltage-dependent	Novel
Potassium inwardly-rectifying, channel	**insulin secretion by glucose pathway suspect**
potassium channel regulator	Novel
T-type calcium channel, voltage-dependent	**Cloned from β-cells, ? role for insulin pathway**
cyclic nucleotide-gated cation channel	Novel
amiloride-sensitive cation channel	Novel
potassium inwardly-rectifying channel	Novel
potassium large conductance calcium-activated channel	Novel
potassium voltage gated channel	Novel
potassium channel subunit	Novel
potassium Calcium-activated channel	Novel
Sodium channel, voltage-gated	Novel
amiloride-sensitive cation channel	Novel
potassium channel	Novel
Inwardly rectifying ATP-sensitive K+ channel (KIR6.2)	**Drug Target via SUR**
Sulfonylurea receptor (SUR2)	**Drug Target for Gliburides (GLUCOVANCE)**

Title: METHODS FOR IDENTIFYING GENES INVOLVED IN GLUCOSE METABOLISM
Inventors: Cheatham et al.
Express Mail Label No. EV192307027US
Atty Docket No. RBN-003PR
Atty: Diana M. Steel
SHEET 81 OF 81

FIG. 17